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Mycobacterium avium subsp. *paratuberculosis* protein MAP 3865c may mimic the pancreatic islet autoantigen Znt8 relevant in Type 1 Diabetes

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Preface

This dissertation is submitted in accordance with the requirements for the PhD degree in Molecular and Biotechnological Science at the Faculty of Biological Science, University of Sassari, Italy. The work was carried out at the Biomedical Science Department, Sassari, Italy. It was supported by grants from The Sardinian Region, PRIN and MIUR.

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To my family and friends, especially Francesca and Paola, thank you for providing a unique support in everything.

Declaration of work done

This thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions. The work was done under the guidance of Professor Leonardo Sechi, at the Department of Biomedical Science, Sassari, Italy.

Riassunto

I fattori ambientali che entrano in gioco nella patogenesi del diabete di tipo 1 (T1D) restano enigmatici. I ruminanti affetti da Paratubercolosi trasmettono il *Mycobacterium avium* subsp. *paratuberculosis* (MAP) all'uomo mediante la contaminazione degli alimenti. MAP è stato ritrovato in un'alta percentuale di pazienti sardi affetti da T1D ma in bassa percentuale nei pazienti affetti da T2D e nei soggetti di controllo. Abbiamo voluto indagare se anticorpi diretti contro epitopi della proteina MAP3865c, altamente omologa alla proteina umana zinc transporter 8 (ZnT8) specifica delle cellule β, potevano cross-reagire con gli epitopi omologhi della ZnT8. A tale scopo è stata saggiata la risposta anticorpale diretta contro la MAP3865 in pazienti sardi affetti da T1D, T2D e nei controlli sani tramite ELISA. Gli anticorpi diretti contro la MAP 3865c riconoscono due epitopi transmembrana immunodominanti nel 52-65% dei pazienti T1D, solo nel 5-7% dei pazienti T2D e nel 3-5% dei controlli sani. È stata riscontrata una correlazione lineare tra titoli di MAP3865c e anti-ZnT8 Abs che riconocono i 2 epitopi omologhi e la preincubazione dei sieri con gli epitopi (peptidi) Znt8 blocca il legame dei peptidi MAP3865c. I risultati dimostrano che gli anticorpi che riconoscono gli epitopi della MAP3865c cross reagiscono con quelli omologhi di Znt8, attraverso un meccanismo di mimetismo molecolare che potrebbe innescare il T1D negli individui infettati da MAP.

Summary

The environmental factors at play in the pathogenesis of type 1 diabetes (T1D) remain enigmatic. *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is transmitted from dairy herds to humans through food contamination. MAP causes an asymptomatic infection which is highly prevalent in Sardinian T1D patients compared with type 2 diabetes (T2D) and healthy controls. Moreover, MAP elicits humoral responses against several mycobacterial proteins. We searched whether antibodies (Abs) against one of these proteins, namely MAP3865c, which displays a sequence homology with the β-cell protein zinc transporter 8 (ZnT8) could be cross-reactive with ZnT8 epitopes. To this end, Ab responses against MAP3865c were analyzed in Sardinian T1D, T2D and healthy subjects using indirect ELISA. Abs against MAP3865c recognized two immunodominant transmembrane epitopes in 52-65% of T1D patients, but only in 5-7% of T2D and 3-5% of healthy controls. There was a linear correlation between titers of anti-MAP3865c and anti-ZnT8 Abs targeting these two homologous epitopes, and pre-incubation of sera with ZnT8 epitope peptides blocked binding to the corresponding MAP3865c peptides. These results demonstrate that Abs recognizing MAP3865c epitopes cross-react with ZnT8, possibly underlying a molecular mimicry mechanism which may precipitate T1D in MAP-infected individuals.

Keywords: autoimmunity, environment, MAP, mimicry, Slc30A8

Contents

1Introduction

1.1 Description of type 1 diabetes mellitus (T1D)

T1D is a T cell-mediated autoimmune disease resulting from the destruction of pancreatic β-cells which produce insulin to proper control blood sugar levels (Akerblom *et al.,* 2002). Lack of insulin causes an increase of fasting blood glucose (around 70-120 mg/dL in nondiabetic people) that begins to appear in the urine above the renal threshold (about 190-200 mg/dl in most people), thus connecting to the symptom by which the disease was identified in antiquity, sweet urine. Glycosuria or glucose in the urine causes the patients to urinate more frequently, and drink more than normal (polydipsia) (WHO, 1999).

T1D is a paradigmatic example of autoimmune disease stemming from a complex interaction between genetic and environmental factors. While several genetic susceptibility loci have been pinpointed by genome-wide association studies (Concannon *et al.,* 2009), the environmental factors at play remain boldly elusive. Yet, environmental factors play a prominent role in T1D pathogenesis, as suggested by the incomplete (~65%) T1D concordance between monozygotic twins (Redondo *et al.,* 2009), by migrant studies (Bodansky *et al*., 1992, Kondrashova *et al.,* 2007) or by the decreasing weight of susceptible and protective HLA Class II haplotypes over the last decades (Hermann *et al.,* 2003, Gillespie *et al.,* 2004).

1.2 Classification of diabetes mellitus

The etiological classification of diabetes and related disorders of glycemia includes, type 1; type 2; gestational diabetes mellitus and those due to specific mechanisms and diseases.

1.2.1 Type 1 diabetes

T1D is a chronic autoimmune disease in which destruction or damaging of the β-cells in the islets of Langerhans results in insulin deficiency and hyperglycemia. Insulin is required for survival, to prevent the development of ketoacidosis, coma and death (WHO, 1999). T1D

account for at least ten percent of all cases of diabetes and develops as a consequence of a combination of genetic predisposition and environmental factors. During the period preceding the clinical onset, autoantibodies (aAb) against antigens present in the pancreatic islets such as insulin, glutamic acid decarboxylase (GAD65), tyrosine phosphatase-like insulinoma antigen-2 (IA-2) and zinc transporter 8 (Znt8) may be detectable for months up to years before hyperglycemia (Orban *et al.*, 2009). Insulin dependent T1D without signs of autoimmunity, where no autoantibodies are found but β-cell destruction is evident, is classified as a type 1 idiopathic diabetes (WHO, 1999). In children the disease process usually is rapid, but more gradual in adults. The slowly progressive form among adults, with phenotypic type 2 diabetes (T2D) but a slowly developing autoimmune process, is referred to as latent autoimmune diabetes in adults (LADA).

1.2.2 Type 2 diabetes (T2D)

T2D is characterized by combinations of decreased insulin secretion and decreased insulin sensitivity (insulin resistance). Exogenous insulin is usually not required for survival (WHO, 1999) and the treatment consist of diet, increased physical activity and weight loss. The symptoms of the hyperglycaemia are usually not severe, but the disease increases the risk of severe late complications.

1.2.3 Gestational diabetes (GD)

GD means pronounced carbohydrate intolerance with onset during pregnancy (especially during third trimester of pregnancy) resulting in hyperglycaemia (WHO, 1999).

1.2.4 Other specific types

Those include less common types where the cause can be identified in a relatively specific manner (WHO, 1999). This group of diabetes includes for example genetic defects on β-cell function or insulin action, e.g. different forms of maturity onset diabetes in the young (MODY). There are also rare cases of diabetes caused by diseases of exocrine pancreas, drug- or chemical induced or caused by infections (ADA, *Report* 1997).

1.3 Epidemiology of type 1 diabetes

T1D incidence has increased intensively over the last two decades, especially in children under the age of five years old (Harjutsalo *et al.*, 2008). There is a wide global variation in the incidence of T1D (Figure 1). The highest incidence is found in Finland (>40/105) and Sardinia (37.8/105) (Borchers *et al.*, 2010). The Scandinavian countries and USA, Canada, Australia and New Zealand are also high-incidence countries, while lower rates are found in the southern Europe. The lowest incidence is found in Venezuela (0.1/105) and China (0.1-4.5/105) and other Asian countries (Borchers *et al.*, 2010).

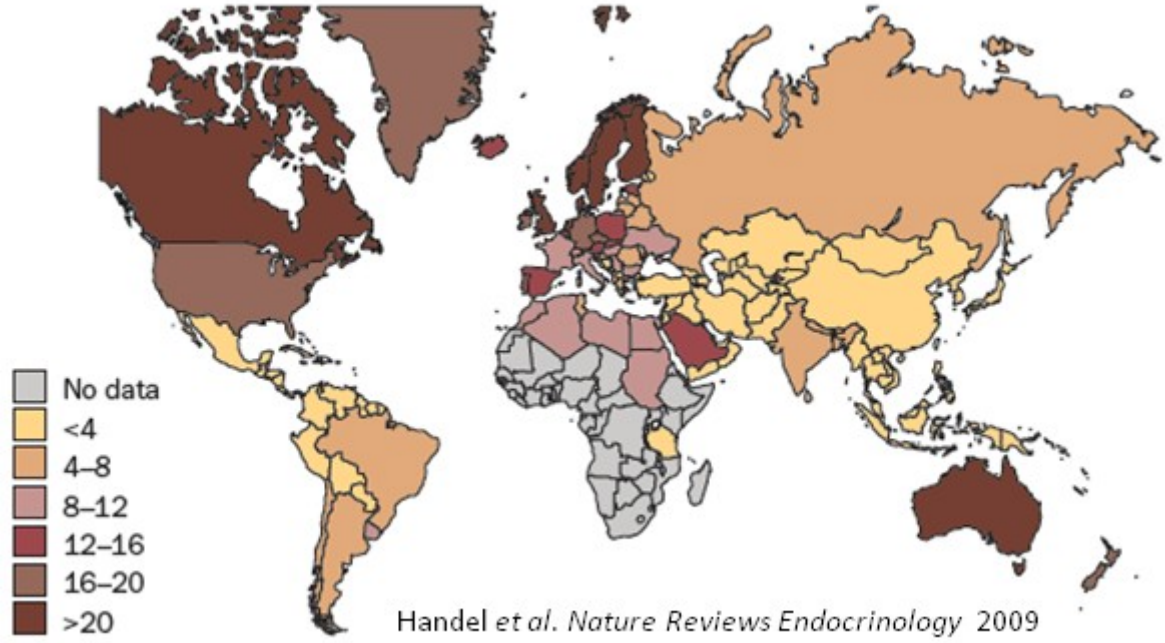

Fig. 1. The global incidence for T1D (among age 0–14 years old children). Modified from Handel *et al.,* Nature Reviews Endocrinology, 2009.

It is unclear to what extent these geographic differences may reflect variation in genetic susceptibility, in prevalence of causal environmental factors, or both.

1.4 Aetiology of type 1 diabetes

The aetiology of T1D is largely unknown but the development is believed to be determined by a combination of genetic susceptibility genes, immune dysregulation and environmental factors. The resulting autoimmune process may be initiated several years before clinical onset of T1D. The autoimmune process causes a decrease in β-cell mass, leading to diminishing insulin production.

T1D is a polygenic disease, in which the genetic background is essential but not sufficient in causing the disease (Patterson *et al.*, 2009). Indeed, approximately 85-90% of new onset T1D patients do not have a first degree relative with the disease, this implies a strong environmental component to contribute to the development of T1D (Akerblom *et al.*, 2002).

1.5 Genetics of T1D

Several genes contribute to T1D susceptibility. The genetic complexity is shown by meta-analyses and genome-wide association studies which indicate that more than 40 loci affect T1D susceptibility (Barrett *et al.*, 2009). Each gene taken individually carries a weak effect, except for the human leukocyte antigen (HLA) region of chromosome 6, encoding the HLA DR and DQ molecules, which is the major locus associated with T1D. The highest risk genotype is DR3-DQ2 (DQB1*0201) and DR4-DQ8 (DQB1*0302) while the allele DQB1*0602 is associated with disease protection (Bluestone *et al.*, 2010). The second most important susceptibility locus on chromosome 11 resides in a variable number of tandem repeat (VNTR) polymorphism in the promoter region of the insulin gene (Bennett *et al.*, 1995, Kennedy *et al.*, 1995). The magnitude of risk correlates with the number of this tandem repeats. These VNTR regulate the insulin expression levels in the thymus by affecting Autoimmune regulator transcription factor (AIRE) binding to its promoter region (Pugliese *et al.*, 1997). There are two main types: VNTR type I will induce lower transcription of insulin and its precursors in the thymus, leading to reduced tolerance and T1D development.

VNTR type III allelic variant, instead confer dominant protection to the individual, as T cells are more efficiently eliminated by negative selection in the thymus.

But also Cytotoxic T lymphocyte associated antigen 4 (CTLA-4) gene polymorphisms on chromosome 2q (Ueda *et al.,* 2003,), as well as other loci (Ladner *et al.*, 2005) have been found to modulate the susceptibility.

1.6 Pathogenesis of T1D

The pathogenesis of T1D is not completely understood. Clinical, autoimmune T1D develops when immune tolerance is broken by environmental factors in genetically predisposed individuals, leading to recognition of specific β-cell antigens by helper T-cells that subsequently activate effector T-cells, and these effector T-cells then directly mediate β-cell killing (insulitis). However, the cellular and molecular pathogenesis of Type 1 diabetes is more complex.

The clinical onset of T1D is preceded by a symptomless and subclinical phase, during which the islet-specific autoimmune process starts and progressively matures. Autoimmune insulitis has been investigated in several animal models like the nonobese diabetic (NOD) mice and BioBreeding (BB) rats greatly enhancing our knowledge in pathogenesis of T1D. Thus, it has been demonstrated that disease occurs as a consequence of defects in immune regulation, which results in expansion of autoreactive $CD4^+$/$CD8^+$ T cells (Bluestone *et al.,* 2010, DiLorenzo *et al.,* 2005).

Upon activation, the T cells expand and migrate from local draining lymph nodes to the islets. Here the immune response is amplified by local antigen-presenting cells (APC) which stimulate the T cells to produce cytokines and chemokines. Other cell types are also involved in the pathogenesis, these includes natural killer (NK) cells (Ogasawara *et al.,* 2003), monocytes, dendritic cells (DC), B cells and cells of the innate immune system (Zipris *et al.,* 2010). The result is an inflammatory infiltrate (insulitis) in which the cells activate and co-stimulate each other. Disease pathogenesis is associated with a shift of the cytokine pattern in the pancreas from an immunological balance (low levels of IFNγ, high levels of IL-10) to a Th1 type (high levels of IFNγ, low levels of IL-10) cytokines (Healey *et al.*, 1995). Moreover, proinflammatory Th17 cells have been described to be involved in T1D, both in animals (Emamaullee *et al.*, 2009) and in humans (Marwaha *et al.,* 2010, Bradshaw *et al.*, 2009). The

subsequent effector mechanisms are not completely clarified. Most likely it is a combination of antigen-specific mechanisms mediated by $CD4^+$ and $CD8^+$ T cells and non-antigen-specific mechanisms as inflammatory cytokines and reactive oxygen species.

These effector mechanisms elicit various signaling pathways in the β-cells resulting in apoptosis and necrosis (Mandrup *et al.*, 2003). Both major histocompability complex (MHC) I- and II-restricted β-cell antigen-specific T cells have been identified in NOD mice and in human T1D patients. Furthermore, disease is characterized by the expansion of B cells (Serreze *et al.*, 1998) producing autoantibodies directed against islet β-cell antigens.

1.7 Autoantibodies against β-cell antigens

The autoimmune response in patients with T1D is often accompanied by the presence of circulating autoantibodies to antigens expressed in the pancreatic β-cells. Autoantibodies to insulin (IAA), the tyrosine-phosphatase like protein (IA-2), the smaller isoform of glutamate decarboxylase (GAD65) and Zinc transporter 8 (ZnT8) alone or in combination are routinely used to predict disease (Sherr *et al.,*2008, Taplin *et al.,* 2008).

Insulin autoantibodies (IAA) are often the first autoantibody to appear in individuals who develop autoimmunity to islet antigens and the levels of IAA correlate inversely with age since levels of IAA decline at older ages (Ziegler *et al.,* 1999). The frequency of IAA positive patients seems to be more common in children from areas with high incidence of T1D and the presence of IAA appears to give a clinically milder disease (Holmberg *et al.,* 2006). Insulin autoantibodies are found in 40-70% of newly diagnosed patients (Holmberg *et al.,* 2006, Williams *et al.,* 2003) and in 0.9-3% of a healthy population (LaGasse *et al.,* 2003, Strebelow *et al.,* 1999).

GAD (glutamic acid decarboxylase) in term of function is rate limiting enzyme that catalyzes the conversion of glutamic acid to γ-amino butyric acid (GABA), an inhibitory neurotransmitter. There are two isoforms of GAD termed GAD-65, GAD-67 that are 76% homologous in amino acid sequence. Indeed only GAD-65 has been shown to be expressed in human islets and is responsible for the autoantibody response. Autoantibodies (aAbs) against GAD-65 are found in about 50-80% of newly diagnosed T1D patients (Holmberg *et al.,* 2006,

Borg *et al.* 2002, Leslie *et al.,* 1999, Bonifacio *et al.,* 1999). In contrast 0-3% of the general population has GAD-65 aAbs (LaGasse *et al.,* 2003, Strebelow *et al.,* 1999 Leslie *et al.,* 1999).

Tyrosine phosphatase–like insulinoma antigen-2 **(**IA-2), a member of the protein tyrosine phosphates family, is a major auto antigen in type 1 diabetes, of all newly diagnosed type 1 diabetic patients, 70% have auto antibodies to IA-2, and these aAbs appear years before the development of clinical disease. The IA-2 molecule is 979 amino acids in length and consists of an intracellular, transmembrane, and extracellular domain. Autoantibody is directed exclusively to the intracellular domain (Zhang *et al.,* 1997). About 55-80% of newly diagnosed T1D patients have aAb against IA-2 (Borg *et al* 2002, Leslie *et al.,* 1999).

ZnT8 is a promising recently discovered autoantigen in type 1 diabetes (Wenzlau, Juhlet *et al.,* 2007, Wenzlau, Moua, Sarkar *et al.,* 2008).

ZnT8 is a 369 amino acids membrane protein and belongs to the Cation Diffusion Facilitator family of highly homologous Znt (Slc30) proteins. In mammalian cell these group of proteins count 10 members named ZnT1 to-10. The Znt (Slc30) proteins allow cellular efflux of Zinc and are characterized by a similar structure consisting of six transmembrane helices and a histidine-rich intracellular loop between helices IV and V (Chimienti *et al.*, 2004).

Znt8 is localized in insulin secretory granules and is specifically expressed in human islet β cells where it facilitates transport of zinc ions from the cytoplasm into insulin secretory vesicles (Chimienti *et al.*, 2006). Znt8 is mainly expressed in pancreatic β cells, but also expressed in the epithelial cells of thyroid although in less quantum. It has also been shown that ZnT8 is present in alpha cells even if zinc is not thought to be required for glucagon processing and packaging (Wijesekara *et al.*, 2010).

This 32 kDa protein is encoded by SLC30A8 (Seve *et al.*, 2004) and aAbs are detected in new-onset T1D patients, most importantly in a sizeable percentage (up to 30%) of T1D patients who otherwise test negative for other aAbs (Wenzlau, Moua, Sarkar *et al.,* 2008). When using all four aAbs combined, more than 96% of Caucasian T1D patients can be identified at disease onset (Wenzlau Juhl *et al.,* 2007).

The use of ZnT8Ab as an independent marker for autoimmune diabetes has been discussed and the aAb can be detected in the prediabetic period, where they are usually preceded by GAD65Ab and IAA (Wenzlau, Juhl *et al.,* 2007, Achenbach *al.,* 2009).

1.8 Environmental factor

A number of observations support the role of the environment in the pathogenesis of T1D:
Firstly, the rise in T1D incidence is occurring more rapidly than can be accounted for by genetic change (Gillespie *et al.*, 2004), thus emphasizing the influence of environmental factors (Karvonen *et al.*, 1993, Variation, 2000).
Secondly, studies on monozygotic twins show a high yet incomplete rate of concordance. 27% in a Finish study (Hyttinen *et al.*, 2003) and 65% in an American one with a longer follow up (Redondo *et al.*, 2008).
Finally studies in transmigratory populations, show that T1D incidence reflects that of the hosting region rather than of the region of origin (Bodansky *et al.*, 1992).
Environmental factors that could play a role in triggering the autoimmune response are briefly described below:

1.8.1 Dietary factors

Dietary factors such as cow milk proteins, cereal proteins and low vitamin D intake (Akerblom *et al.*, 2002).
Associations between cow's milk exposure and β-cell autoimmunity and later T1D development was shown in several studies (Virtanen *et al.*, 1993, Kimpimaki *et al.*, 2001).
Cow's milk has been proposed to cause autoimmunity because of reported cross-reactivity between serum antibodies against albumin and ICA-1, a β-cell surface protein (Karjalainen *et al.*, 1992). Further data should be provided to asses a pathogenic role for Cow's milk protein.
Early introduction of gluten as well, has been reported as a risk factor for β-cell autoimmunity (Ziegler *et al.*, 2003). Gluten and related cereal proteins ingestion can trigger autoimmunity in susceptible individuals, whom develop celiac disease (CD). CD is an inflammatory intestinal disease, resulting in villus atrophy. Overlap between CD and T1D has long been acknowledge

and may be partially explained by the sharing of HLADQ2/DQ8 predisposing genotype (Sollid *et al.*, 1989).

Immune reactivity to both Cow's milk and wheat proteins in T1D could be the consequence of an aberrant mucosal response rather than the source (force) of islet autoimmunity.

Vitamin D has been shown to have immune-modulatory, specifically immunosuppressive, characteristics. It may down-regulate the autoimmune process that leads to overt diabetes (Deluca 2001). The north–south gradient in the disease incidence of T1D may be correlated with lower mean monthly sunshine hours, which in turn may correlate with decreased biologically active vitamin D3 (EURODIAB, 1999). It has been shown that a polymorphism within the vitamin D receptor initiation codon or within the vitamin D receptor gene is linked to increased risk for disease (Pani 2000 *et al.,* Ban *et al.*, 2001). If uptake of vitamin D is decreased by a defective receptor, dysregulation of T-cell-mediated activity could occur.

1.8.2 Viral infection

Viruses are thought to act against β cells by at least two mechanisms. The first is by direct cytotoxicity to the β cells; the second is by triggering an autoimmune process that targets the β-cells (Yoon *et al.,* 1991).

The role of enteroviruses (eg. Coxsackievirus B4 and rotavirus) has been thoroughly investigated (Tracy *et al.,* 2010). NOD mouse model suggests that enteroviral infection may accelerate rather than initiate T1D progression because they are effective only once autoimmune T cells have already accumulated in the islets (Serreze *et al.,* 2000).

For infectious exposures, the hygiene hypothesis proposes that increased hygiene may cause changes in the composition of gut bacterial flora (Wen *et al.*, 2008), influencing the maturation of the immune system, facilitating imbalance and thereby autoimmune reactions in genetically predisposed individuals (Ludvigsson *et al.,* 2006). Thus suggesting that exposure to a large number of infections early in life prevents development of autoimmunity due to an appropriately priming of the adaptive immune system (Stracha *et al*., 1989). Infections, especially in early childhood, may in fact prevent or delay disease (Bach *et al.*, 2001). In

some studies, the frequency of childhood infections correlated inversely with the incidence of T1D (Pundziute *et al.*, 2000, Gibbon *et al.,* 1997).

1.8.3 Bacteria

The bacterial composition of the intestine has long been acknowledged as an important variable affecting T1D development. Direct evidence exists in rodents, for example, feeding probiotic bacterial strains, usually lactic acid bacteria, to non-obese diabetic (NOD) mice or biobreeding diabetes-prone (BB-DP) rats can delay or prevent diabetes (Matsuzaky *et al.,* 1997*,* Calcinaro *et al.,* 2005, Yadav *et al.*, 2007). Feeding antibiotics to NOD mice or BB-DP rats can also increase survival in these models (Brugman *et al.*, 2006; Schwartz *et al.*, 2007).
In addition, pathogen-free NOD mice lacking an adaptor protein for multiple toll-like receptors known to bind to bacterial ligands fail to develop diabetes (Wen *et al.*, 2008). Perhaps autoimmunity ensues whenever the intricate microbial balance in the intestine is disturbed. Additionally, the intestinal wall does not seem to have the same capacity to form a coherent barrier separating luminal bacteria and the immune system in T1D models and patients versus controls. This so-called "leaky gut" phenotype is thought to enhance the exposure of bacterial antigens to the immune system (Vaarala *et al.*, 2008). In the intestine of T1D patients, subclinical immune activation (Westerholm *et al.,* 2003) and evidence for an impaired regulatory T cells (Treg) subset (Tiittanen *et al.*, 2008) were found. Treg are a specialized subpopulation of T cells which suppresses activation of the immune system and thereby maintains tolerance to self antigen.
Thus, both antibiotics and probiotics may influence T1D development by altering the balance of gut microbiota toward either a tolerogenic or nontolerogenic state, depending on constitution of the intestinal microflora at the time of administration (Vaarala *et al.,* 2008).
Another recently discovered bacterial risk factor maybe *Mycobacterium avium* subspecies *paratuberculosis* (MAP).

1.9 MAP – the organism

MAP belongs to the family of Mycobacteriaceae, which also includes the tuberculosis and leprosy-causing species *Mycobacterium tuberculosis* and *Mycobacterium leprae* respectively (Wayne and Kubica, 1986). Mycobacteria are defined by their acid-fast properties, having a cell wall containing mycolic acids, and a high (61-71%) genomic C+G content (Levy *et al.,* 1992). There are now 153 established and validated species and 11 subspecies of Mycobacteria http://www.bacterio.cict.fr. MAP is a member of the *Mycobacterium avium complex* (MAC), which comprises four named subspecies: *M. avium* subsp. *avium, M. avium* subsp. *silvaticum, M. avium* subsp. *paratuberculosis* and *M. avium subsp hominissuiss*. These subspecies represent phenotypically diverse organisms, ranging from environmental bacteria that cause opportunistic infections of swine and immune-compromised people to professional pathogens of birds and ruminants (Turenne *et al.,* 2008). In vitro doubling time vary between subspecies from 12 to over 20 hours, with MAP being the slowest grower (over 48 hours) of the four subspecies. MAP is rod-shaped microorganism of 0.5 to 1.5μm in size. A MAP primary culture from veterinary/clinical or food specimens can take 3–4 months or longer. However, once established, a MAP isolate will produce colonies within 3–6 weeks upon subculture at their optimum growth temperature of 37°C under aerobic conditions. MAP forms small (1–2 mm) non-pigmented or pigmented rough colonies white and domed with an entire margin; rough colonies are rarely seen. The organism has a requirement for the incorporation of the iron-chelating compound mycobactin J into any complex medium used for its cultivation (Merkal and McCullough, 1982). This mycobactin dependency represents a characteristic unique to MAP. Another unique identifier is the insertion element IS900 that occurs as 14–18 copies within the genome of MAP (McFadden *et al.,* 1987, Green *et al.,* 1989). This IS900 element forms the basis of molecular detection methods for MAP such as PCR assays.

1.10 Pathogenesis of MAP

MAP is an obligate pathogenic parasite of animals; the only place it can multiply in nature is in a susceptible host, within macrophages (Collins, 2003). When MAP leaves an animal, for example in faeces or milk, it can survive for extended periods in soil and water, but it is unable to multiply outside the host. Consequently, the primary source of infection with MAP is an infected animal. There is evidence that MAP can exist in vegetative, cell wall deficient (Chiodini *et al.,* 1986), and dormant (Whittington *et al.,* 2004) forms.

Natural infections with MAP primarily occur via the fecal-oral route. The bacteria are ingested mainly via feed, water or teats that are contaminated. Following the ingestion of MAP*,* the bacteria are transported from the intestinal lumen into the intestinal wall via M cells, which overlie the domes of Peyer's patches (Momotani *et al.,* 1988). It is believed that the fibronectin attachment protein (FAP) expressed by MAP facilitates MAP targeting and invading M-cells through the formation of a fibronectin bridge between FAP on MAP and integrins on the apical surface of intestinal M cells (Secott *et al.* 2001, Secott *et al.,* 2002).

After crossing the epithelial lining of the intestine, the bacteria are phagocytized by subepithelial macrophages (Bendixen *et al.,* 1981). MAP preferentially resides in phagosomes or early endosomes of host macrophages, predominately those associated with ileal Peyer's patches. In severe cases, MAP may be found in macrophages distributed throughout a number of tissues in infected animals (Gwozdz *et al.,* 1997, Koenig *et al.,* 1993), although even in these cases, the majority of organisms are found associated with macrophages infiltrating intestinal tissues and adjacent draining lymph nodes (Hines *et al.,* 1995).

Once within the macrophage, MAP avoids the bactericidal action of the professional phagocytic cells and proliferates. The various mechanisms that enable mycobacteria to survive within phagosomes include inhibition of phagosomal maturation, resistance against antimicrobial molecules, and adaptation to host-induced metabolic constraints (Kuehnel *et al.,* 2001). From time to time lysed bacteria are processed and presented to T-lymphocytes. The proinflammatory mediator released by lysate macrophage, account for a massive infiltration of CD4 effector T-cells. T- cell-mediated immune responses are essential in determining the outcome of infection and overall severity of disease. Infections with MAP usually persist in a

subclinical state for several years (Kennedy *et al.,* 2001, Manning *et al.* 2001, Storset *et al.*, 2001) until development of clinical signs, the latter being largely due to chronic inflammation of infected tissues.

1.11 MAP and Johne's disease

MAP is the established causative agent of a debilitating chronic granulamatous enteritis in cattle and other domestic and wild ruminants, termed Johne's disease (JD) or Paratuberculosis (Chiodini *et al.*, 1984). Susceptible animals include cattle, sheep, goats, but animals such as red deer (Godfroid *et al.*, 2000), rabbits (Greig *et al.*, 1999) and other non-ruminant wildlife species (Beard *et al.*, 2001) can also be affected. When an animal is exhibiting clinical symptoms of Johne's disease (persistent diarrhea, weight loss, progressive emaciation), and comes to the attention of the farmer, it has reached the clinical stage of the disease. A major concern with JD is that in the intervening years between infection and clinical manifestation of Johne's disease, clinically or subclinically infected animals shed MAP in feces and milk, enabling dissemination to susceptible domestic ruminants, the environment, and, in retail milk (Sweeney *et al.*, 1992, Streeter *et al.*, 1995). JD is prevalent in sheep, goat and cattle herds at differing levels throughout the world. Not only the bacterium is shed in the milk of infected cows but it also survives pasteurization. A study in the United Kingdom by Grant *et al.* (2002) tested 244 raw milk samples and 567 commercially pasteurized samples. These authors detected MAP by PCR in 7.8% of raw milk samples and 11.8% of pasteurized samples and they were able to culture MAP from 1.6% of raw samples and 1.8% of pasteurized samples (Grant *et al.,* 2002). Consumption of contaminated milk could expose individuals to potentially pathogenic bacteria that may cause serious health problems (Millar *et al.,* 1996). The main problem with JD in many respects is that it represents a hidden threat for farmers. Although most animals are infected at an early age, the onset of clinical symptoms is usually delayed by several years (typically 2–5 years) (Pozzato *et al.,* 2011).

1.12 MAP and Crohn's disease

MAP has been associated with Crohn's disease (CD) in humans, a chronic inflammatory bowel disease of the gastrointestinal tract, which is characterized by general malaise, chronic weight loss, abdominal pain, and diarrhea (Chiodini *et al.*, 1989). Although the distal ileum is most commonly involved, CD may affect any part of the gastrointestinal tract. It is a life-long disease that has no known cure and sufferers may have a poor quality of life because of pain and, in extreme cases, uncontrolled discharge of intestinal content (Hermon-Taylor, J. and Bull, T. 2002). It is believed that CD has a complex and multifactorial aetiology.

Crohn's disease could be due to: (i) a persistent infection, possibly involving mycobacteria (specifically MAP); (ii) a defective mucosal barrier (leaky gut) which allows uptake of bacterial, dietary and other immunogenic macromolecules; (iii) dysregulation of the host immune response with loss of tolerance, aggressive cellular activations and disorders of apoptosis; (iv) genetic susceptibility factors; or (v) a combination of some, or all, of the above (Shanahan *et al.*, 2002, Sarton *et al.*, 2003). Mutations in a gene on chromosome 16, known as NOD2/CARD15, are associated with Crohn's disease (Hampe *et al.*, 2001, Hugot *et al.*, 2001, Ogura *et al.*, 2001). Three major mutations of this gene are most commonly encountered in Crohn's patients – R702W, G908R and L1007 fsinsC (Lesage *et al.*, 2002). The proportion of cases of Crohn's disease that can be attributed to NOD2/CARD15 mutations has been estimated at 15–30%, which would be consistent with roles for a number of other factors, both genetic and environmental, in the pathogenesis of Crohn's disease (Newman *and* Siminovitch, 2003, Sechi *et al*., 2005 JCM). Those data provides compelling evidence to support the hypothesis that Crohn's disease results from bacterial insult in genetically susceptible individuals. NOD2/CARD15 serves a role in bacterial recognition, activating the nuclear factor (NF) κB pathway. Cellular studies have documented that NOD2/CARD15 is a cytosolic protein activated by muramyl dipeptide, a degradation product of bacterial peptidoglycan (Girardin *et al.*, 2003, Inohara *et al.*, 2003).

MAP has sporadically been isolated from humans with Crohn's disease by decontamination and culture of resected tissue specimens and incubation for very long periods (>1 year).

Chiodini *et al.* (1984) were the first to isolate an initially unclassified *Mycobacterium* sp., later definitively identified as MAP (McFadden *et al.,* 1987), from resected terminal ileum of three young patients with Crohn's disease. The three strains, designated Linda, Ben and Dominic, had taken up to 18 months for primary isolation. It was later reported that all three strains had originally been isolated as nonacid-fast coccobacillary forms that had the ultrastructural appearance of sphaeroplasts (cell wall deficient forms) which, after several months incubation, transformed into characteristic MAP -like organisms (Chiodini *et al.*, 1986). This would explain why acid-fast cells are not readily observed in sections of Crohn's tissue by histological examination. Moreover studies applying *in situ* hybridization with IS900 based DNA probes to Crohn's tissue have revealed the presence of MAP DNA in higher percentages in Crohn's tissue compared with controls (Sechi *et al.*, 2001, 2004). These studies confirm that MAP is present in Crohn's disease tissue in a spheroplast (cell wall deficient) form which is unable to retain the acid-fast stain, and hence is not visible upon Ziehl Neelsen staining (Sechi *et al*., 2005 Am J G), whereas MAP in Johne's specimens have cell walls intact.

Since the initial isolations by (Chiodini *et al.*, 1986), MAP has been successfully cultured from resected tissue of further Crohn's patients in various parts of the world (USA, UK, The Netherlands, Australia, France, Italy and the Czech Republic),(Schwartz *et al.*, 2000, Sechi *et al.*2005, Bull *et al.,* 2003) and also from breast milk of mothers suffering from Crohn's Disease. This can expose infants to MAP very early in life, a significant concern if they are genetically susceptible to developing Crohn's Disease (Naser *et al.*, 2000). MAP has as well been cultured from the blood of CD patients (Naser *et al.*, 2004). It is also concerning that MAP has been found in milk and meat taken from infected cattle (Mutharia *et al.*, 2010).

1.13 MAP and T1D

A possible association between MAP and T1D has been recently proposed, mainly because quite a few line of evidence point out that MAP can be at play in triggering autoimmunity towards pancreatic β-cells (Sechi *et al.,* 2008, Paccagnini *et al.,* 2009).

Evidence supporting a link between MAP and T1D includes: higher detection rates of MAP by IS 900 specific PCR in samples from T1D patients compared with controls; demonstration of a serological response to MAP antigens and whole cell lysates in T1D patients (Rosu *et al,*. 2008). MAP isolation from the blood of Sardinian patients affected by T1D but not in those with T2D (Rosu *et al.,* 2009).

Moreover the founding of a relevant SLC11A1 (ex NRAMP1) gene polymorphisms in T1D patients (Paccagnini *et al.,* 2009) previously reported in Crohn's patients (Sechi, Gazouli *et al.,* 2006), suggests indeed that there is a strong possibility of MAP being involved with autoimmune responses in T1D just in the same manner as it did in Crohn's. NRAMP1 (Natural Resistance-Associated Macrophage Protein) is an iron transporter associated with macrophage activation. This gene has multiple pleiotropic effects on macrophage function, including regulation of cytokine production, tumor necrosis factor α, interleukin-1 b, inducible nitric oxide synthase and regulation of major histocompatibility complex class II (MHC-II) expression and antigen presentation functions (Blackwell *et al.,* 1999, Dai *et al.,* 2009). All of these activities are not only essential for protection against mycobacterial infection (innate defenses), but also critically involved in the induction and progression of autoimmune diseases. Since MAP persists within macrophages and is processed by dendritic cells (DC), mutant forms of SLC11A1 may alter the processing or presentation of MAP antigens leading to diabetogenic responses (Dai *et al.,* 2009).

Counting ~1.8 million inhabitants, ~3.5 millions sheeps and approximately two hundred thousand cattle, MAP exposure may be particularly elevated in the Western Mediterranean island of Sardinia, where it is estimated that ~60% of flocks may be contaminated. Sardinia is also one of the regions with the highest incidence of T1D and multiple sclerosis (MS) worldwide, a notable exception in the north-south gradient followed by these autoimmune diseases (Figure2).

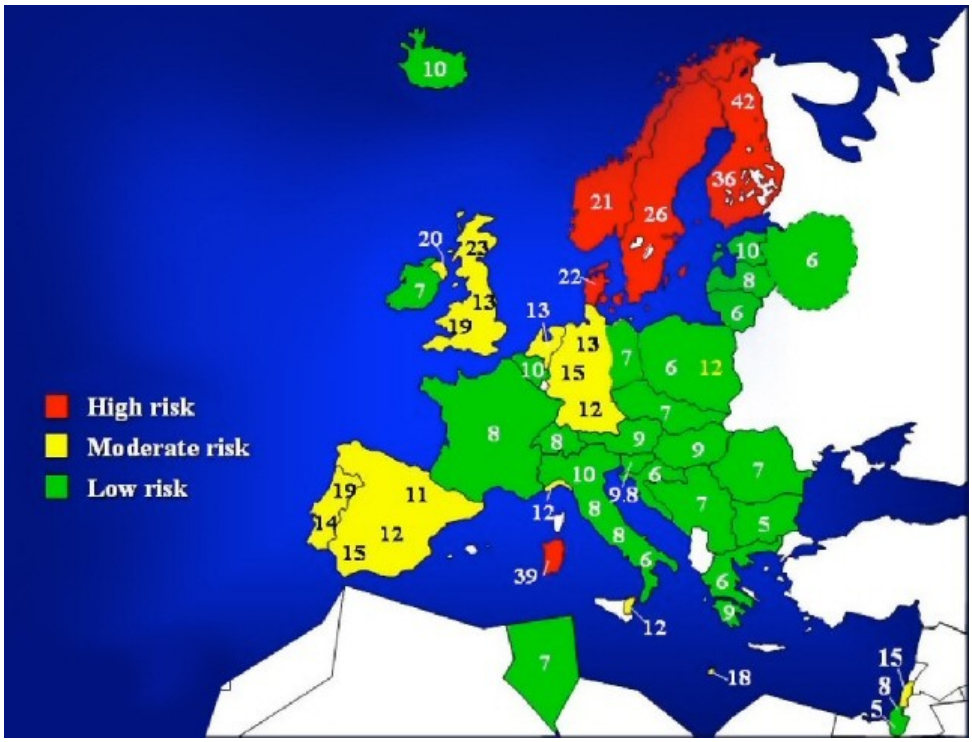

Fig. 2. Incidence of T1DM in children aged <15 years from 20 centers, distributed across 17 countries in Europe. Data from EURODIAB (the Epidemiology and Prevention of Diabetes) study group.

Although evidence for a cause-effect relationship is lacking, MAP transmission to humans has long been associated with Crohn's disease both in Sardinia (Sechi, Gazouli *et al.*, 2005, Di Sabatino *et al.*, 2011) and elsewhere (Naser *et al.*, 2004). The hypothesis stating that MAP infection may be a potential candidate environmental trigger also for T1D is based on two key findings. First, MAP infection is highly prevalent in Sardinian T1D patients. Indeed, MAP DNA can be isolated from blood in 63% of Sardinian T1D patients, but only in 16% of healthy controls (Sechi, Rosu *et al.,* 2008); the MAP envelope protein MptD can be detected in the blood of 47.3% Sardinian T1D patients, but in a smaller proportion of T2D patients (7.7%) and healthy controls (12.6%) (Rosu *et al.,* 2009); and MAP bacilli can be cultured

from blood (Rosu *et al.,* 2009). Second, this MAP infections triggers a specific humoral response, as Sardinian T1D patients display high frequencies of antibodies (Abs) reacting against mycobacterial proteins (heparin-binding hemagglutinin, glycosyl transferase, whole MAP lysates (70% Ab+ T1D patients *vs.* 7.6% Ab+ healthy controls) (Sechi, Rosu *et al.,* 2008) and the MAP-specific proteins MptD and MAP3738c when compared to T2D and healthy controls (Sechi, Rosu *et al.,* 2008).

In this work, we investigated whether another MAP protein, namely MAP3865c, may be involved in a similar molecular mimicry mechanism. Antigenic molecular mimicry is defined by cross-reactive immune responses because of significant structural homologies shared by molecules encoded by dissimilar genes. Either linear amino acid sequences of the molecules or their conformational epitopes may be shared, even though their origins are separate. The disease pathogenesis may involve multiple factors including the genetics of the host, strain of MAP, activation status of the autoreactive T cells, upregulation of pancreatic MHC class I antigens, molecular mimicry between MAP and β cell epitopes and T-cell mediated β-cells destruction by cytotoxic mechanism (Davies *et al.,* 1997). MAP, as one of the environmental factors affecting the induction of T1D, may act as triggering agents of autoimmunity or (less probably) as primary injurious agents, which directly damage pancreatic β cells. Immune responses against a determinant shared by host cells (β- cells) and MAP could cause a tissue-specific immune response by generation of cytotoxic cross-reactive effector lymphocytes or antibodies that recognize self-proteins located on the target cells. Notably, it has already been reported as a case of cross-recognition between the mycobacterial heat shock protein 65 (hsp65) and the self auto-antigen, glutamic acid decarboxylase (GAD65), involved in T1D patients (Scheinin *et al.,* 1996). Here it was demonstrated that antibodies against MAP 3865c are capable of cross-reacting with host determinants.

It was shown that Sardinian T1D patients specifically mount anti-MAP3865c Abs responses. Two Abs epitopes were identified in the MAP3865c sequence and shown to be homologous to the β-cell antigen zinc transporter 8 (ZnT8) (Chimienti *et al.,* 2004) targeted by auto-Abs in T1D patients (Wenzlau, Juhl *et al.*, 2007). Anti-MAP Abs recognizing these regions were found to be cross-reactive with the homologous ZnT8 sequences, raising the possibility of a

molecular mimicry between mycobacterial and β-cell epitopes.

2 Aim of the Study

The study was conducted in order to identify specific antigens of an environmental pathogen such as MAP, which might play a major role in the pathogenesis of T1D. MAP might be an infectious agent at play in the proliferation of autoreactive T-cell directed against pancreatic islets. The first goal of this study was to identify islet cell protein homologues to MAP proteins which may be putatively relevant in molecular mimicry. After Basic Local Alignment Search Tool (BLAST) homology search, the highest scoring candidate was MAP 3865c which showed high similarity to the human ZnT8 (zinc transporter 8; Slc30A8). In view of the above, MAP3865c from *Mycobacterium avium* subsp. *paratuberculosis* was cloned into pMALc2X and expressed in *E.coli* TB1. MAP3865c and selected MAP3865c epitopes were then assessed by indirect ELISA for serological reactivity. Competition assays were as well performed. We wanted to demonstrate that Abs against MAP3865c epitopes cross-react with Znt8 epitopes, possibly underlying a molecular mimicry mechanism which may precipitate T1D in MAP-infected individuals. The investigation performed on T1D, T2D patients and HC suggests that MAP infection may be a potential candidate environmental trigger for T1D. All the data reported on this thesis derive from studies conducted on a Sardinian population cohort. It remains to be seen whether this finding will be confirmed by others in different patient cohorts.

3 Material and methods

3.1 Patient and control serum samples

T1D patients (n=34; mean age 34.5±7.7 years, mean age at onset 17.5±10.2 years, mean T1D duration 16.8±9.9 years) and T2D patients (n=56; mean age 64.8±8.6 years, mean age at onset 56.4±9.2 years, mean T2D duration 8.5±5.3 years) diagnosed according to the American Association of Diabetes criteria (Association AD. 2011) and healthy blood donors (n=63) age-matched with T1D patients (mean age 38.5±12.0 years; p=0.102) were recruited at the University Hospital of Sassari. Written informed consents were obtained before blood drawing. Serum samples were processed soon after the drawing. Briefly, blood from patients was centrifuged, and serum supernatants were used for enzyme-linked immunosorbent assay (ELISA); the remaining sera were aliquoted and stored frozen at -20°C for short-term storage (<6 months) and -80°C for long-term storage (>6 months).

3.2 MAP IS900 amplification

The presence of MAP-specific DNA in blood samples was detected by PCR amplification of IS900 sequences. Briefly: Two rounds of amplification were undertaken. Firstly primers Liz1 and Liz2, secondly primers Av1 and Av2 described in Table 1, were used to amplify a 298-bp fragment specific for *Mycobacterium avium* subsp. *paratuberculosis*. The reaction mixture (final volume of 50 µl) comprised primers at a concentration of 0.5 µM, Expand High Fidelity reaction buffer (1µ), 200 µM (each) dNTPs, and 3 Uof Expand High-Fidelity Taq polymerase (Expand High-fidelity PCR system; Roche, Lewes, United Kingdom). For cycling conditions see Table 1. Amplified fragments were visualized with ethidium bromide on 2 % agarose-1000 gel (Life Technologies, Grand Island, NY) and purified with a QIAquick gel extraction kit (QIAGEN, Crawley, United Kingdom). Each amplicon was then sequenced in both directions by using Av1 and Av2 primers.

Table 1. Schematic overview of IS900 Nested PCR's steps.

IS900 PCR primers	Cycling conditions
Liz1: 5'- CTTTCTTGAAGGGTGTTCGG-3'	94°C for 2 min and 36 cycles of 94°C
Liz2: 5'- ACGTGACCTCGCCTCCAT-3'	for 40 s, 58°C for 40 s, and 72°C for 40 s
(First amplification, amplicon length 298bp)	final step 72°C for 5 min
Av1: 5'-ATGTGGTTGCTGTGTTGGATGG-3'	94°C for 2 min and 36 cycles of 94°C
Av2: 5'- CCGCCGCAATCAACTCCAG-3'	for 40 s, 62°C for 40 s, and 72°C for 40 s
(Second amplification, amplicon length 298bp)	final step 72°C for 5 min

3.3 Construction of the pMAL-MAP3865c expression vector

MAP DNA was extracted with the detergent cetyltrimethylammonium bromide (Sigma). The full-length *MAP3865c* gene was amplified by PCR from the MAP DNA ATCC43015 with a sense primer (5'-GCGCGAATTCATGGGCGCCGGCCACAACCACAC-3') and an antisense primer (5'- GCGCCTGCAGTCATCAGAAGCTGTCGGAGCACTC-3'), where underlined sequences are *Eco*RI and *Pst*I restriction sites, respectively. Because the DNA has an average GC content of 69%, Dimethyl sulfoxide (up to 5%) was used to aid in amplification. The amplified fragment of 914 bp was inserted into the pMAL-c2x vector (New England Biolabs) between *Eco*RI and *Pst*1 cleavage sites next to a maltose-binding protein (MBP) sequence Figure 3. This plasmid has an inducible lacZ promoter, an exact deletion of the malE signal sequence and a MCS. Expression vector pMAL-c2 and the PCR product were double digested with *Eco*RI and *Pst*I enzymes and purified with the Qiaquick PCR purification kit.

The ligation mix was used to transform *E. coli* K12 TB1 frozen competent cells. One hundred µL of ligation mixture was added to 1ml of LB medium and incubated at 37° C. After 1 hour 100 µL of the grown culture were spread on a LB plate containing 100 mg/L ampicillin and incubated at 37° C overnight. Colonies were picked with a sterile toothpick and inoculated

onto a master LB amp plate and a LB amp plate containing 80 mg/L *Xgal* and 0.1 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG). The colonies were incubated at 37 °C for 14 h and the white clones on LB amp plate containing *Xgal* and IPTG were selected. The corresponding patches on the master plate were determined.

To confirm the correct gene fusion between mal*E* and *MAP 3865c*, plasmid DNA was miniprepared from a single ampicilin-resistant colony. The insert of *MAP 3865c* was identified by restriction enzyme digestion and DNA sequencing.

The coding sequence of the cloned *MAP3865c* gene fully matched the published sequence of the *MAP3865c* gene of *M. paratuberculosis* K10 (GenBank accession number: NC002944) (Li, Bannantine *et al.,* 2005).Ligated constructs resulted in an "in-frame" fusion between the *malE* gene in the vector, which encodes MBP, and the *MAP3865c* gene. MBP is the N-terminal located affinity tag.

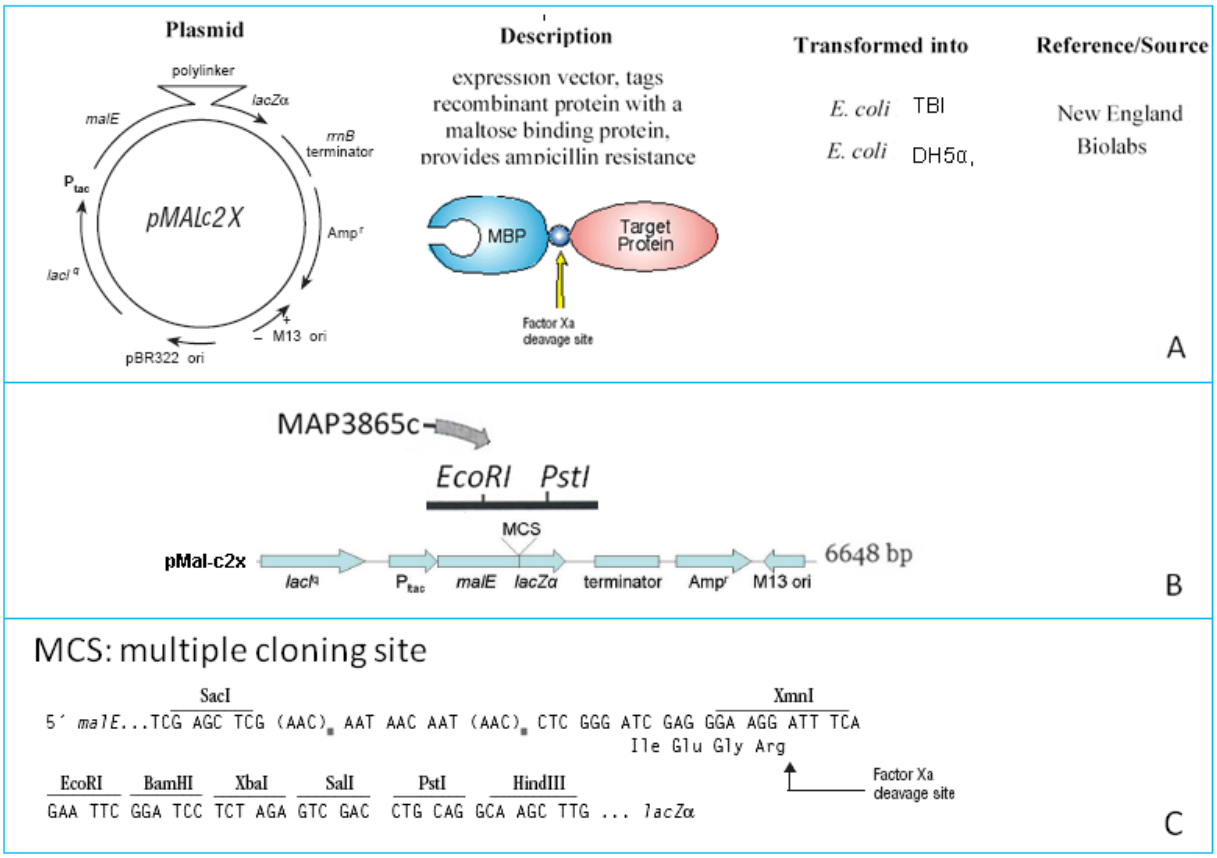

Fig. 3. (A) Relevant features of pMal-c2x, (New England Biolabs). This vector is used for expression of recombinant maltose binding protein-tagged proteins in *E. coli* hosts such as TB1 and DH5α. This vector counts an ampicillin resistance gene (AmpR) as selectable marker. (B) pMAL-c2x is shown as linear for display purposes. The cloned gene is inserted downstream from the *malE* gene of *E. coli*, which encodes maltose-binding protein (MBP) resulting in the expression of an MBP fusion protein. The *malE* gene is fused to the *lacZ*α gene. Restriction sites between *malE* and *lacZ*α are available for inserting the coding sequence of interest. Insertion inactivates the β-galactosidase α- fragment activity of the *malE-lacZ*α fusion, allowing a blue-to-white screen for inserts on X-gal plates. (C) Magnification of the polilinker (MCS), unique restriction sites are indicated.

3.4 MAP3865c protein expression and purification

E. coli TB1 cells harboring the expression plasmid were grown at 37°C and a single colony was used to inoculate 1 liter of rich medium containing 100 µg/ml ampicillin and 2 g/l glucose. Cells were grown at 37°C with shaking until they reached an OD600 of approximately 0.5. MBP- MAP3865c fusion protein expression was induced by addition of 0.3 mM IPTG (Sigma After 2 h, cells were harvested, resuspended in 20 ml of column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1:100 Sigma protease inhibitor cocktail) and frozen at –20°C.

The following day, cells were lysed by sonication in short pulses of 15 s (for about 2 to 3 min), with 15 s of cooling between each sonication. Supernatants (crude extracts) were diluted 1:5 with column buffer, loaded on a column charged with amylose resin (New England Biolabs) and washed 5 times. The fusion protein was eluted with column buffer containing 10 mM maltose by collecting fractions of 0.5 ml. To assess protein yield, purity, and size, the eluted fractions containing protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue (Sigma) staining. The MBP-MAP3865c fusion protein migrated at the expected molecular mass of 72.5 kD.

Fractions containing the purified MBP-MAP3865c protein were pooled and concentrated by using Centricon centrifugal filter devices (Millipore). Purified protein from the control strain consisted of an MBP fusion of the LacZ alpha peptide. *Map3865c* gene sequence was inputted

into DNA Star program. The sequence was translated into protein and its antigenic feature was calculated.

3.5 Peptidic Library

The heptapeptide library spanning the amino acid sequences of MAP3865c (Table 3) was obtained from GenScrip The Biology CRO,Piscataway, NJ 08854, USA.

Stock solutions were prepared at 10 mM in DMSO and stored in single-use aliquots at -80°C.

Table 2. Sequences of the 60 peptides included in the Library

Peptide	Sequence
80412_1	MGAGHNH
80412_2	NHTPAET
80412_3	ETGDARL
80412_4	RLIPRMV
80412_5	MVMAAAI
80412_6	AILAAFF
80412_7	FFVVELV
80412_8	LVTSLLI
80412_9	LINSIAL
80412_10	ALLADAG
80412_11	AGHMLTD
80412_12	TDVVAVF
80412_13	VFMGLAA
80412_14	AAVTLAR
80412_15	ARGSSSP
80412_16	SSPARTY
80412_17	TYGWHRA
80412_18	RAEVFTA
80412_19	TAVANAG
80412_20	AGLLIGV
80412_21	GVSVFIL
80412_22	ILYEAIQ
80412_23	IQRLREA
80412_24	EAPAVPG

80412_25	PGVPMIA
80412_26	IAVALAG
80412_27	AGLAANF
80412_28	NFVVALL
80412_29	LLLRSHS
80412_30	HSSGSLA
80412_31	LAVKGAY
80412_32	AYLEVIA
80412_33	IADTVGS
80412_34	GSLGVLI
80412_35	LIAGVVT
80412_36	VTVTTRW
80412_37	RWPYADV
80412_38	DVVVAVL
80412_39	VLVALWV
80412_40	WVLPRAI
80412_41	AISLARD
80412_42	RDALRIL
80412_43	ILSESSP
80412_44	SPTHIDV
80412_45	DVEELRA
80412_46	RAALGAV
80412_47	AVDGVTG
80412_48	TGVHDLH
80412_49	LHVWTLS
80412_50	LSPGKDM
80412_51	DMCTAHL
80412_52	HLISTGD
80412_53	GDSARVL
80412_54	VLRDARA
80412_55	RAVLSAR
80412_56	ARGLAHA
80412_57	HATVQID
80412_58	IDCPDDT

80412_59	DTECSDS
80412_60	TECSDSF

3.6 Peptides designed upon Library Screening and

Peptides MAP3865c$_{125-133}$ (MIAVALAGL) and MAP3865c$_{133-141}$ (LAANFVVAL) along with their respective homologous peptides ZnT8$_{178-186}$ (MIIVSSCAV) and ZnT8$_{186-194}$ (VAANIVLTV) were synthesized at >85% purity (GL Biochem). Conserved amino acids residues are underlined. Stock solutions were prepared at 10 mM in DMSO and stored in single-use aliquots at -80°C.

3.7 Enzyme-linked immunosorbent assays (ELISAs)

Indirect ELISA assays were setup to detect Abs specific for MAP3865c protein and peptides. Ninety-six-well Nunc immunoplates were coated overnight at 4°C with 5 µg/ml of recombinant MBP-MAP3865c fusion protein or 10 µg/ml of peptides diluted in 0.05 M carbonate–bicarbonate buffer, pH 9.5 (Sigma). Plates were then blocked for 1 h at room temperature with 5% non-fat dried milk (Sigma) and washed twice with phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBS-T). Serum samples were subsequently added at 1:100 dilution in PBS-T for 2 h at room temperature. After 5 washes in PBS-T, 100 µl of alkaline phosphatase-conjugated goat anti-human immunoglobulin G polyclonal Ab (1:1000; Sigma) was added for 1 h at room temperature. Plates were washed again 5 times in PBS-T and 200 µl of paranitrophenylphosphate (Sigma) added as substrate for alkaline phosphatase. Plates were incubated at 37°C in the dark for 3-6 min and the absorbance at 405 nm read on a VERSA Tunable Max microplate reader (Molecular Devices). Negative control wells were obtained by incubation of immobilized protein or peptides with secondary Ab alone, and their mean values subtracted from all samples. Positive control sera were also included in all experiments. Results are expressed as means of triplicate 405 nm optical density (OD) values.

3.8 Competition assays

Competition assays were performed by pre-incubating sera overnight at 4°C with saturating concentrations (5-20 μM, titrated for each individual serum) of ZnT8 peptides, the corresponding MAP peptides (positive controls), irrelevant peptide (MAP3865c$_{211-217}$, ILSESSP), no peptide, or MAP3865c-MBP fusion protein, as described elsewhere (Mallone *et al.,* 2001). Sera were then subjected to ELISA on plates coated with MAP3865c-MBP, MAP3865c$_{125-133}$ or MAP3865c$_{133-141}$, as above.

3.9 Statistical analyses

Receiver operator characteristic (ROC) curves were used to score the performance of each single ELISA in discriminating T1D or T2D patients from healthy controls. The area under the ROC curve (AUC) was calculated assuming a non-parametric distribution of results. Thus, an AUC of 1.0 would indicate that the assay achieved 100% accuracy in identifying patients; an AUC of 0.5 would indicate that the assay gave no difference between patients and controls; and an AUC of 0 would indicate that the assay gave a positive result for controls and a negative result for patients. The cut-off for positivity in each assay was set at ≥93% specificity (i.e. Ab+ healthy controls ≤7%) and the corresponding sensitivity (i.e. percent of Ab+ patients) calculated accordingly. Clinical characteristics of Ab+ and Ab-negative patients were compared using the Mann-Whitney U test.

4 Results

4.1 IS900 PCR

A total of 29 out of 46 patients were observed positive by PCR (63%), based on the amplification of IS900, a specific signature element within the genome of MAP, whereas, only 8 out of the 50 samples in the control group were observed positive (16%),(Figure 4). Statistical analysis by Chi-square test generated a value of 20.442 with 1 degree of freedom. The two-tailed P value was equal to 0.0001; the association was thus found very significant.

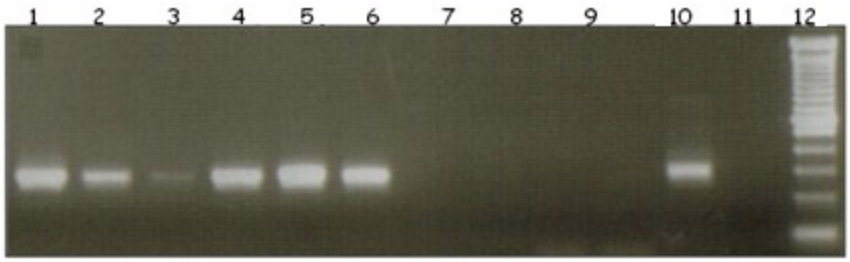

Fig. 4 PCR products from the IS900 (Liz/AV) nested PCR obtained with DNA from the following sources: plasma from patients with T1D (lane 1-6) and from healthy controls (lane 7-9). Lane 10, positive control (298bp amplicon). Lane 11, negative control. Lane 12, 100bp ladder.

4.2 Sequence Analysis

A MAP specific gene was identified by comparisons between the MAP genome (Li *et al.*, 2005) and the repertoire of human genes by using NCBI/BLAST. After BLAST-P analysis, the highest scoring candidate identified was MAP 3865c which share sequence homology to the ZnT8 (zinc transporter 8; Slc30A8) (Figure 5 amino acids region 2–366).

GENE ID: 169026 SLC30A8 | solute carrier family 30 (zinc transporter), member 8 [Homo sapiens] Length=369

Score = 91.3 bits (225), Expect = 4e-23, Method: Compositional matrix adjust. Identities = 87/319 (27%), Positives = 159/319 (50%), Gaps = 25/319 (8%)

```
Query  2    GAGHNHT---PAETG---DARLIPRMVMAAAILAAFFVVELVTSLLINSIALLADAGHML  55
            G  H H+   P E G    A    ++  A+AI   F + E+V   +  S+A++ DA H+L
Sbjct  49   GMYHCHSGSKPTEKGANEYAYAKWKLCSASAICFIFMIAEVVGGHIAGSLAVVTDAAHLL  108

Query  56   TDVVAVFMGLAAVTLARRGSSSPARTYGWHRAEVFTAVANAGLLIGVSVFILYEAIQRLR  115
             D+ +  + L ++ L+ +  S    T+GWHRAE+  A+ +   +  V+  ++Y A +RL
Sbjct  109  IDLTSFLLSLFSLWLSSKPPSK-RLTFGWHRAEILGALLSILCIWVVTGVLVYLACERLL  167

Query  116  EAP-AVPGVPMIAVALAGLAANFVVALLLRSHSSG--------SLAVKGAYLEVIADTVG  166
                  +    MI V+   +AAN V+ ++L     G        + +V+ A++  + D
Sbjct  168  YPDYQIQATVMIIVSSCAVAANIVLTVVLHQRCLGHNHKEVQANASVRAAFVHALGDLFQ  227

Query  167  SLGVLIAG-VVTVTTRWPYadvvvavlvalwvlPRAISLARDALRILSESSPTHIDVEEL  225
            S+ VLI+  ++     +  AD +   + ++ VL   I++ +D   +L E  P  ++   +
Sbjct  228  SISVLISALIIYFKPEYKIADPICTFIFSILVLASTITILKDFSILLMEGVPKSLNYSGV  287

Query  226  RAALGAVDGVTGVHDLHVWTLSPGKDMCTAHLIS--TGDSARVLRDARAVLSARGLAHA-  282
            +  + AVDGV  VH LH+W+L+  + + +AH+ +  + DS  V R+    LS     H+
Sbjct  288  KELILAVDGVLSVHSLHIWSLTMNQVILSAHVATAASRDSQVVRREIAKALSKSFTMHSL  347

Query  283  TVQIDCPDDTE-----CSD  296
            T+Q++ P D +     C D
Sbjct  348  TIQMESPVDQDPDCLFCED  366
```

Fig. 5. Blast alignment obtained using GenBank non-redundant proteins database with low complexity region filter. Result shows region of identity, strong similar amino acid with a plus (+) and a missing region in dashes (-). Query sequence is MAP 3865c.

MAP 3865c protein sequence was inputted into DNAstar program in order to calculate its antigenic features. Analysis of predicted antigenic attributes of MAP3865c by the DNAStar is shown in Figure 6. The values indicating antigenic index, hydrophilicity and surface probability are seen therein. Possibly significant epitope can be identified on the basis of both antigenic index and the probability to be exposed on the surface of the membrane.

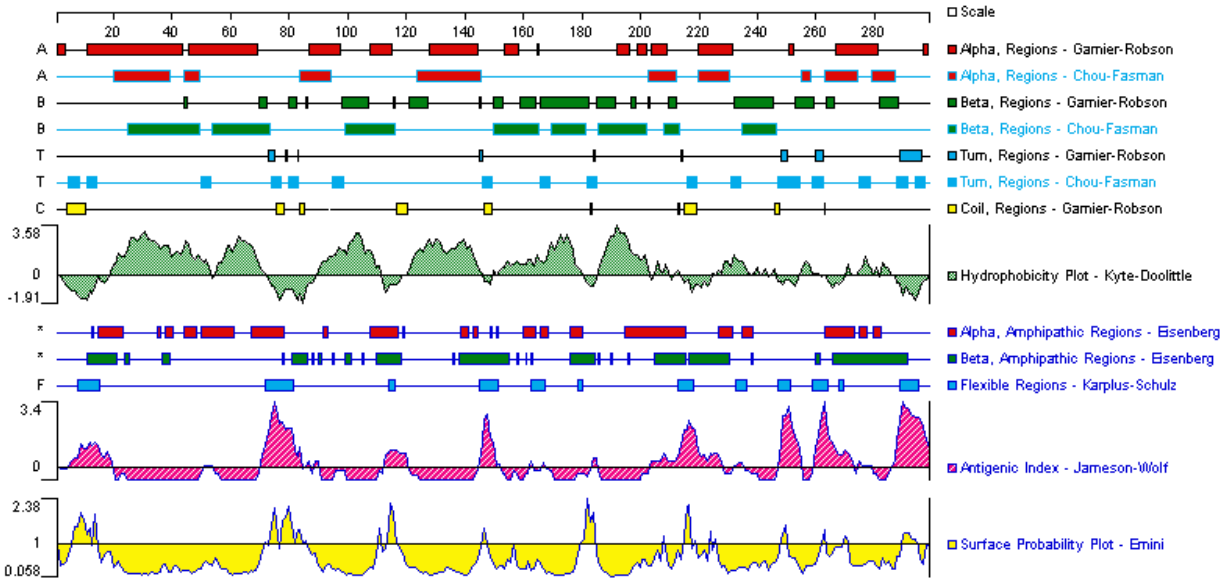

Fig. 6. Computational prediction of the MAP3865c structure and properties such as different helices and turns, hydrophobicity, antigenicity and surface probability, etc. using Protean software from the DNAstar package (DNAstar Inc., Madison, USA).

4.3 Construction of recombinant plasmid

Recombinant plasmid restriction enzyme digestion is shown in Figure 7. The target fragment of 900 bp *MAP 3865c* was inserted into the pMAL-c2x between *Eco*RI and *Pst*I.

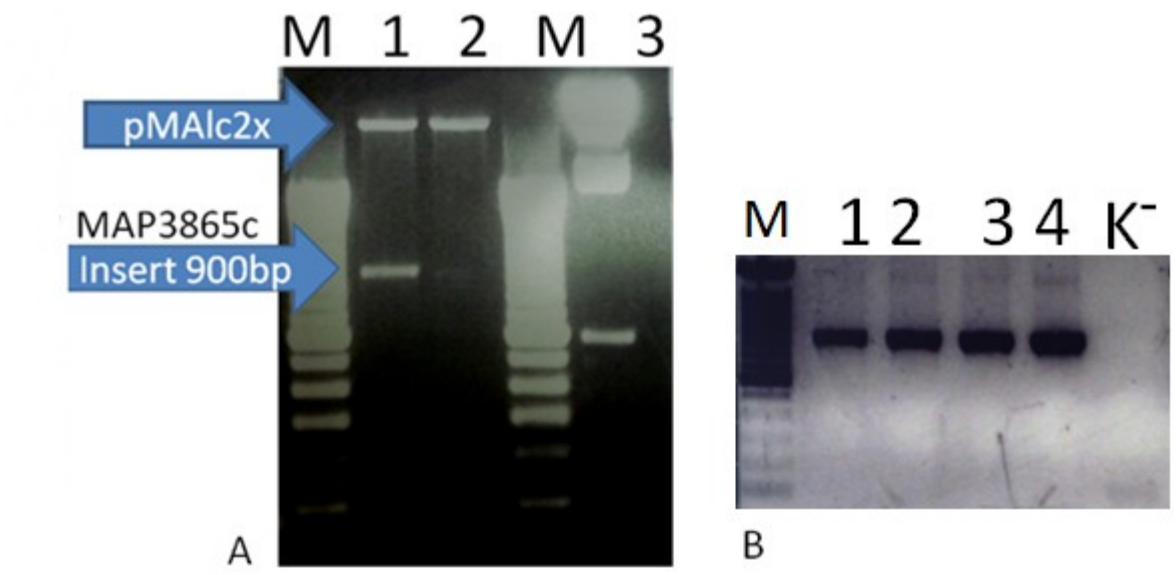

Fig. 7. (A) Restriction analysis of pMAL-c2x- *Map 3865c*. Lane M: 100bp DNA ladder; Lane 1: pMAL-c2x- *Map 3865c* digested by *Eco*RI and P*st*I; Lane 2: pMAL-c2x digested by *Eco*RI; Lane 3: λ Hind III marker. (**B)** Colony PCR. Lane M: 100bp DNA ladder; Lane 1, 2, 3 and 4: *MAP 3865c* PCR fragment. Lane K^-: Negative control (PCR mix without DNA).

4.4 MBP-MAP3865c expression and purification

SDS-PAGE analysis of expressed and purified MBP-MAP3865c is shown in Figure 8. The MBP-MAP3865c fusion protein migrated at the expected molecular mass of about 72,5kD, and the protein yield after affinity purification was 1 mg/ml culture assessed by Thermo Scientific NanoDrop™ 2000.

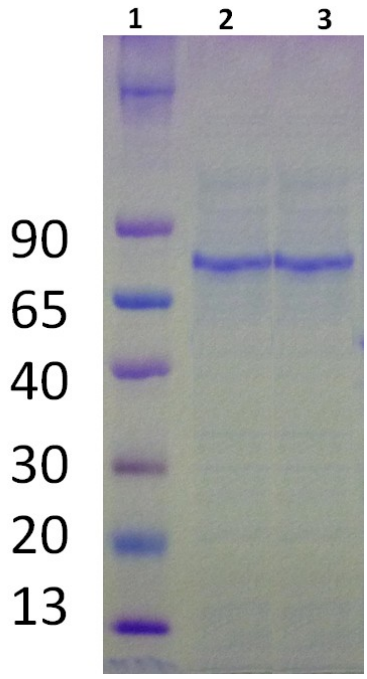

Fig. 8. 10% Bis/Tris (MES) SDS-PAGE analysis of MAP3865c. Lane 1: Color burst ™ molecular weight marker Lane 2 and 3: affinity chromatography purified MBP-MAP3865c.

4.5 Anti-MAP3865c Abs are highly prevalent in Sardinian T1D patients, but not in T2D patients

The purified MBP-MAP3865c fusion protein was first used to screen by indirect ELISA for the presence of serum anti-MAP3865c Abs. As shown in Fig. 9A, 29.4% of T1D patients displayed serum reactivity against MAP3865c compared to 6.4% of healthy controls (AUC 0.68, p=0.014). This reactivity was specific of T1D patients, as it was not significantly different between T2D patients and controls (Fig. 9B; 3.6% vs. 2.9%; AUC 0.55; p=0.396).

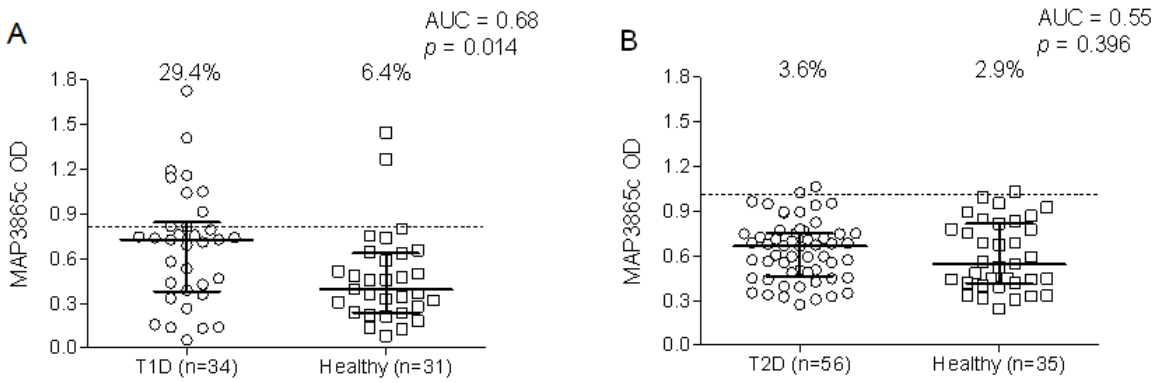

Fig. 9. Prevalence of anti-MAP3865c Abs in Sardinian T1D and T2D patients. Sera were tested for their reactivity against plate-coated MBP-MAP3865c fusion protein. Ab distribution is shown for T1D (A) and T2D (B) patients compared to healthy controls. Dotted lines indicate the cut-off for positivity used in each assay, as calculated by ROC analysis. The percent fraction of Ab+ sera is indicated on top of each distribution, while bars indicate the corresponding median ± interquartile range. AUC and *p* values are given in the top right corner. Figure shows a representative experiment out of three performed.

Since the MAP3865c protein was fused with MBP, Ab+ and Ab-negative sera were tested against the LacZ-MBP control to exclude potential MBP-specific reactivities. A difference in Ab reactivity between Ab+ and Ab-negative sera and between T1D and healthy subjects was only observed when testing with the MAP-MBP protein, while the LacZ-MBP protein did not discriminate any positive samples using the same sera (Fig. 10A).

The ELISA assay employed displayed good reproducibility. For determination of intra-assay variability, a serum with MAP3865c Ab reactivity near the cut-off values was tested 20 times in a single experiment, giving a coefficient of variation of 2.8%. (Fig.10B). The same serum tested in 4 separate experiments yielded an inter-assay coefficient of variation of 7.4% (Fig. 10B).

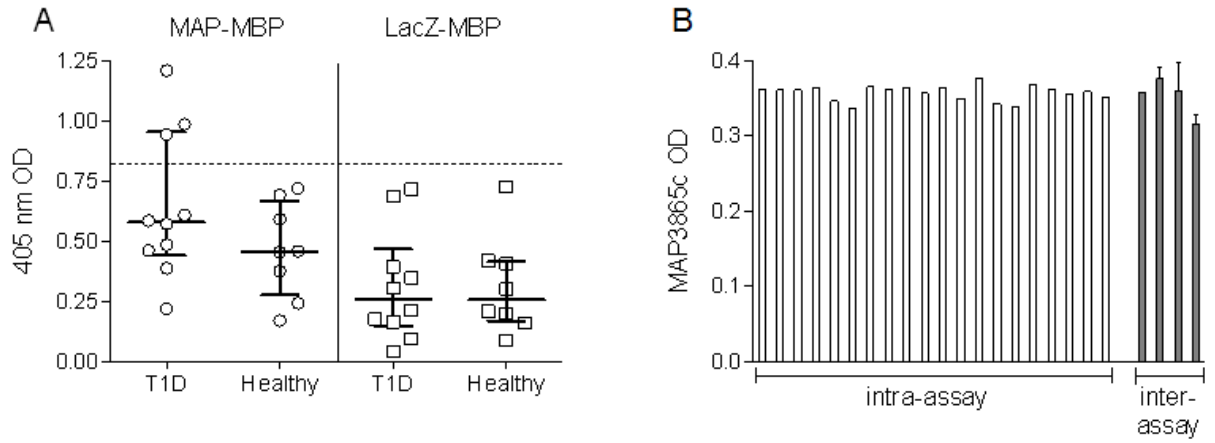

Fig.10. (A) Reactivity against the MAP-MBP fusion protein is MAP-specific. Ab+ and Ab- negative sera from T1D and healthy donors were challenged either with the MAP-MBP fusion protein (as in Fig. 9) or with a LacZ-MBP control protein. The dotted line indicates the cutoff for positivity. (B) Intra- and inter-assay variability of MAP3865c ELISA Ab assays. For intra-assay variability (white bars), the same serum was tested in 20 replicate wells; bars show readouts of each single well. Coefficient of variation is 2.8%. For inter-assay variability (grey bars), the same serum was tested in 4 separate experiments; bars show mean ± SEM of triplicate wells from each experiment. Coefficient of variation is 7.4%.

As previously reported (Sechi *et al.* 2008, Paccagnini *et al.*, 2009), the presence of MAP-specific IS900 DNA was also more prevalent among T1D patients (55.9%) than among T2D and healthy controls (7.0% and 20.0%, respectively; $p<0.001$). However, there was no correlation between positivity for anti-MAP3865c Abs and IS900 DNA (Table 3), although the frequency of Ab+ T1D patients was higher in the IS900 DNA+ group (7/34¸20.6% vs 3/34, 8.8%).

	IS900 PCR+	**IS900 PCR-**
MAP3865c Ab+	20.6%	8.8%
MAP3865c Ab-	52.9%	17.7%

Table 3. Prevalence of MAP-specific IS900 DNA and of anti-MAP3865c Abs in

theperipheral blood of T1D patients (n=34).

4.6 Peptidic library

In order to identify specific epitopes of MAP3865c protein recognized by the people involved in the study an eptamer-peptide-library was screened against 43 T1D patient blood samples (Figure11). The grid in figure 11 summarizes the results obtained. To note patients, who have raised a positive PCR test, are marked in red while the PCR negative T1D subjects are marked in black.

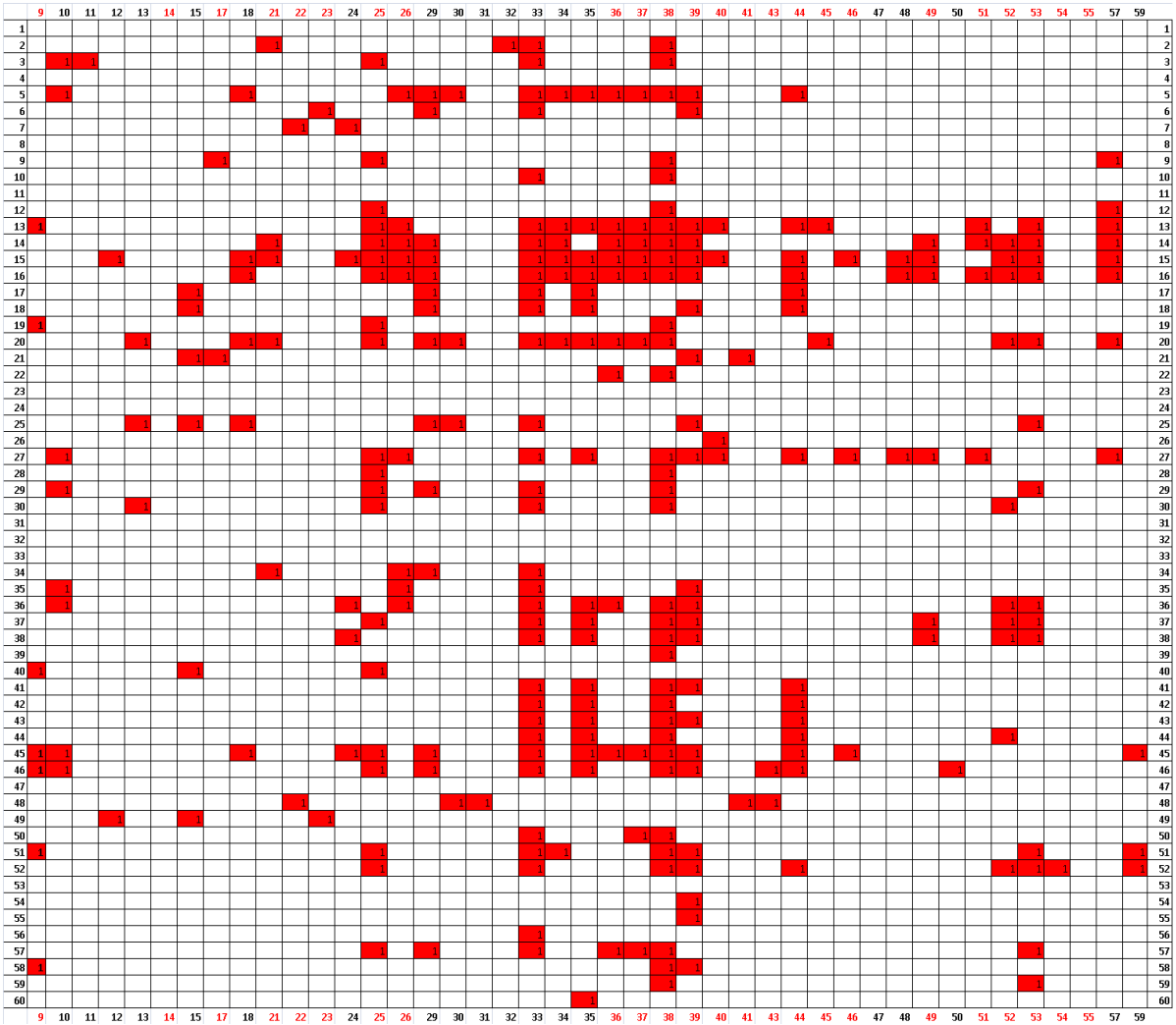

Fig. 11. Grid displaying a positive OD value (in red), versus a non positive OD (in white), for all the sixty 7-mer peptides tested by indirect ELISA.

Testing of the human sera with overlapping synthetic peptides showed that different peptides were highly recognized, indeed 49 out of 60 peptides cross reacted with blood samples of diabetic patients. Among them, peptide 25 PGVPMIA (MAP3865c$_{121-127}$) and 27 AGLAANF (MAP3865c$_{131-137}$) gave the strongest values.
For both peptides, the average OD value of the patients was double compared to the one of the healthy controls. In fact, Peptides 25 PGVPMIA and 27 AGLAANF gave both strong ELISA values (cut-off value of 0.2 calculated by the mean plus two time standard deviation of the mean) with a chi square of 4.548, P <0.033 and 3.678, P <0.027, respectively.

The performance of the ELISA was also assessed by the area under the receiver operating characteristic curve (AUC-ROC) (Figure 12), where the AUC and the p values are given in the top left corner. Figure shows a representative experiment out of three performed.

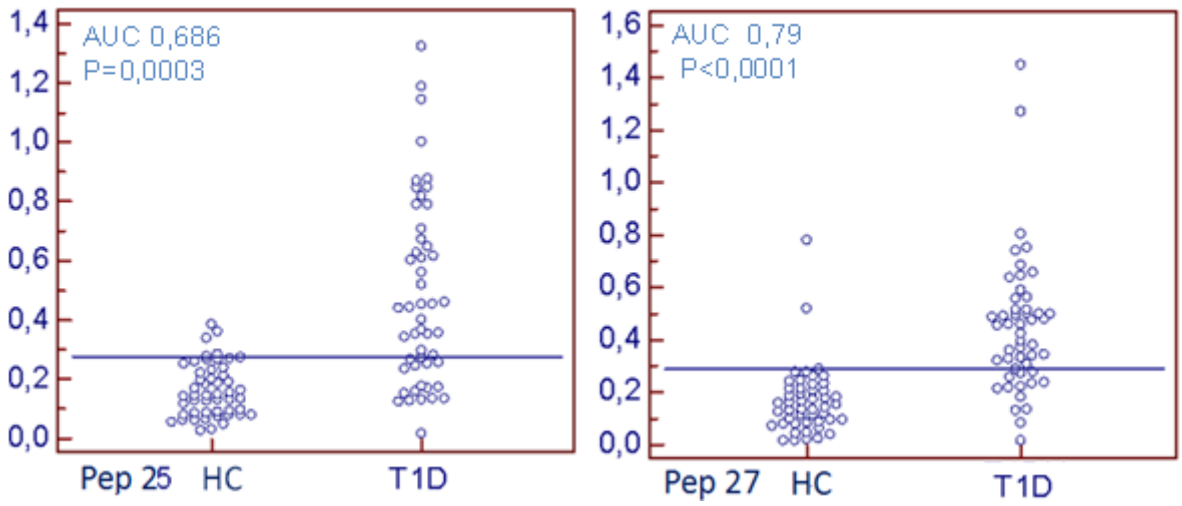

Fig. 12. Evaluation of serum samples from 41 T1D patients right column, and 48 healthy controls (left column, 0) against MAP3865c recombinant protein by the AUC-ROC test.

Peptide 25 PGVPMIA (MAP3865c$_{121-127}$) and peptide 27 AGLAANF (MAP3865c$_{131-137}$), are separated only by three amino acid and share respectively two and three residues with the homolog human protein.

To asses if MAP3865c epitopes recognized by the people involved in the study could be cross-reactive with the homolog Znt8 ones, we looked for cooperation with a group, who works with human Znt8. We started to collaborate with Roberto Mallone, an endocrinologist that studies T1D since long time. His group mainly deals with T-lymphocytes trying to identify immunogenic epitopes. After starting a common research we discovered indeed that the peptides identified by ELISA, peptide 25 PGVPMIA (MAP3865c$_{121-127}$) and peptide 27 AGLAANF (MAP3865c$_{131-137}$), were partially homolog to the peptides identified by Mallone. Noteworthy both MAP3865c$_{125-133}$ (MIAVALAGL) and MAP3865c$_{133-141}$ (LAANFVVAL) epitopes were capable to stimulate $CD8^{+}$T cell responses.

4.7 Anti-MAP3865c Abs recognize an immunodominant transmembrane region homologous to ZnT8.

Scanning of the MAP3865c aminoacid sequence unraveled a 27.5% sequence identity with the human β-cell protein ZnT8 (Slc30A8) (Fig. 13A). To note the peptides which gave significant results are found on one of the highly conserved regions (41.2% aminoacid identity) corresponding to the MAP3865c$_{125-141}$ and ZnT8$_{178-194}$ sequences. These sequences are located in one of the 6 membrane-spanning domains of the two proteins (Fig. 13B).

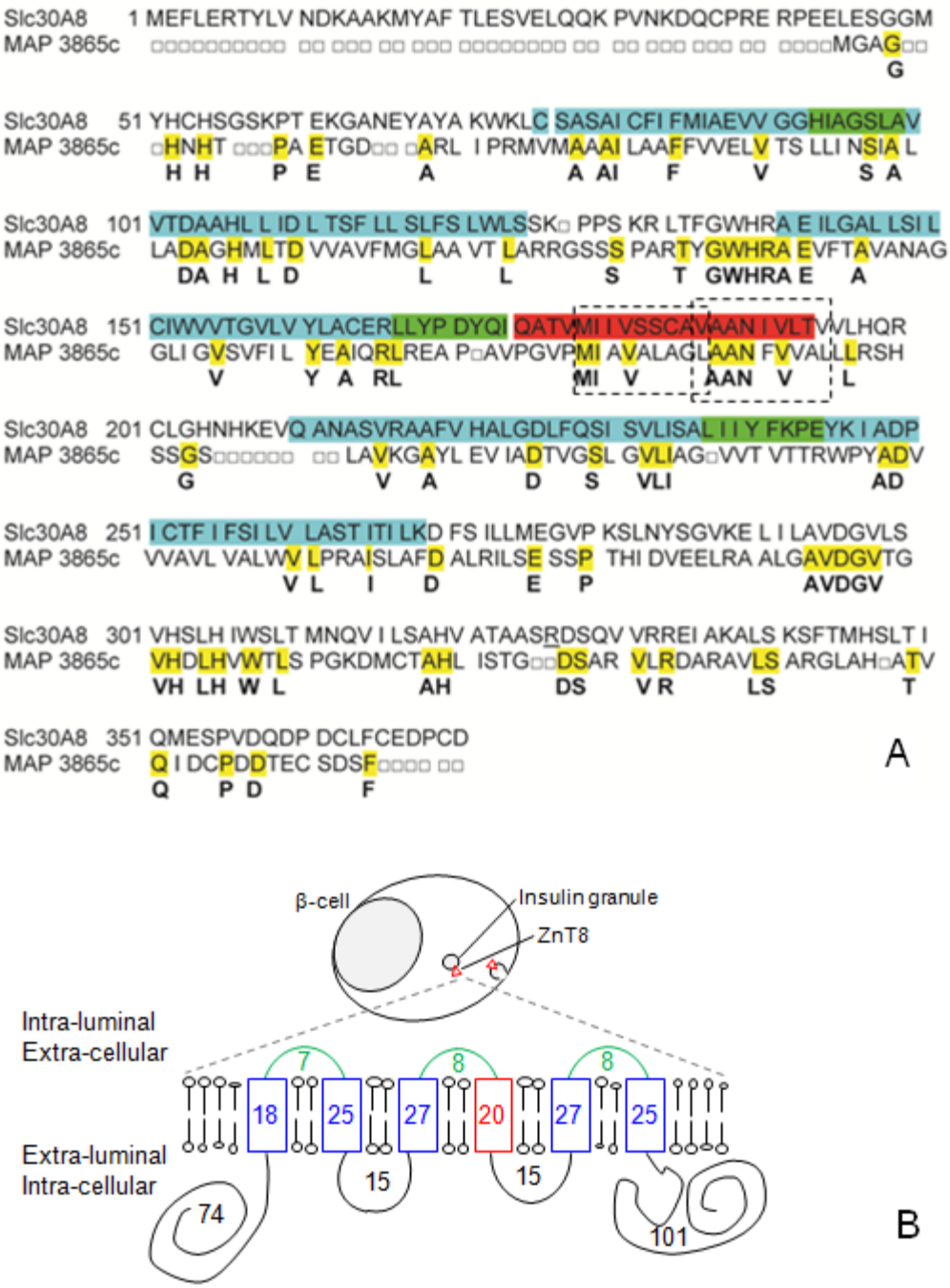

Fig. 13. (A) Aminoacid sequence alignment of ZnT8 (Slc30A8) and MAP3865c proteins. Conserved aminoacid residues are highlighted in yellow within the MAP3865c sequence and listed in bold below the two sequence alignment rows. The other color codes refer to the ZnT8

protein structure shown in (B): sequences highlighted in green belong to the 3 intra-luminal loops; the sequence in red belongs to the fourth transmembrane domain, while those in blue belong to the other transmembrane regions; sequences not highlighted fall within the 4 extra-luminal fragments. Dotted rectangles show the MAP3865c$_{125-133}$/ZnT8$_{178-186}$ and MAP3865c$_{133-141}$/ZnT8$_{186-194}$ peptides studied in subsequent experiments. The topology of the ZnT8 protein is also shown in panel (B), where the 3 intra-luminal loops in green become extracellularly exposed once the granule is released. Conversely, the 4 extra-luminal domains in black are exposed to the cytosol and remain intracellular upon insulin exocytosis.

To further explore the significance of this homology, four nonamer peptides covering this region were synthesized: MAP3865c$_{125-133}$ (MIAVALAGL) and its homologous ZnT8$_{178-186}$ (MIIVSSCAV); and MAP3865c$_{133-141}$ (LAANFVVAL) and its homologous ZnT8$_{186-194}$ (VAANIVLTV). Competition assays demonstrated that these epitopes are immunodominant Ab targets within the full-length MAP3865c protein, as sera pre-adsorbed with these peptides, either alone or in combination, were capable of blocking binding to the MAP3865c-MBP fusion protein, to a similar extent to what observed when pre-adsorbing sera with the MBP-MAP3865c protein itself (Fig. 14).

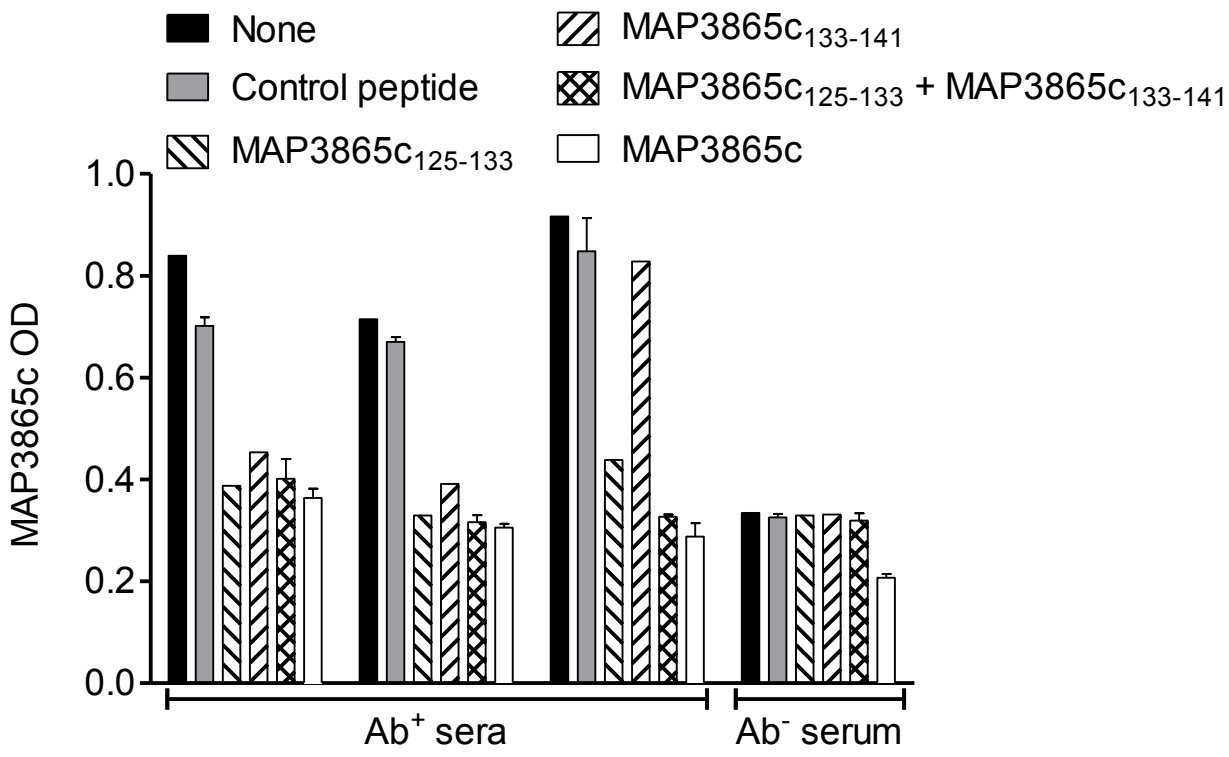

Fig. 14. Ab reactivities against the MAP3865c protein are inhibited by MAP3865c$_{125-133}$ and MAP3865c$_{133-141}$ peptides. Ab+ and Ab-negative sera from T1D patients were pre-incubated overnight with saturating concentrations (5.5 μM) of MAP3865c$_{125-133}$, MAP3865c$_{133-141}$, the two peptides in combination, MAP3865c-MBP fusion protein and control or no peptide. Their reactivity on MBP-MAP3865c-coated ELISA plates was subsequently tested. Bars depict means ± SEM of triplicate wells and results are representative of three separate experiments.

Serum Ab reactivity against these four MAP3865c and ZnT8 peptides was further tested using the same ELISA assay. Also in this case, a significant difference in the frequency of Ab+ sera was observed between T1D and healthy subjects (Fig. 15). The homologous MAP3865c$_{125-133}$ and ZnT8$_{178-186}$ peptides (Fig. 15A-B) were recognized by 65.4% and 68.0% of T1D patients, but only in 4.2% of healthy controls (AUC 0.85 and 0.86, respectively; p<0.0001 for both). This serum Ab reactivity was also observed for the MAP3865c$_{133-141}$ and ZnT8$_{186-194}$ peptides, as 51.6% and 55.6% of T1D patients were Ab+, respectively, compared to 4.2% of healthy controls (AUC 0.75 and 0.79; p=0.0003 and p<0.0001, respectively).

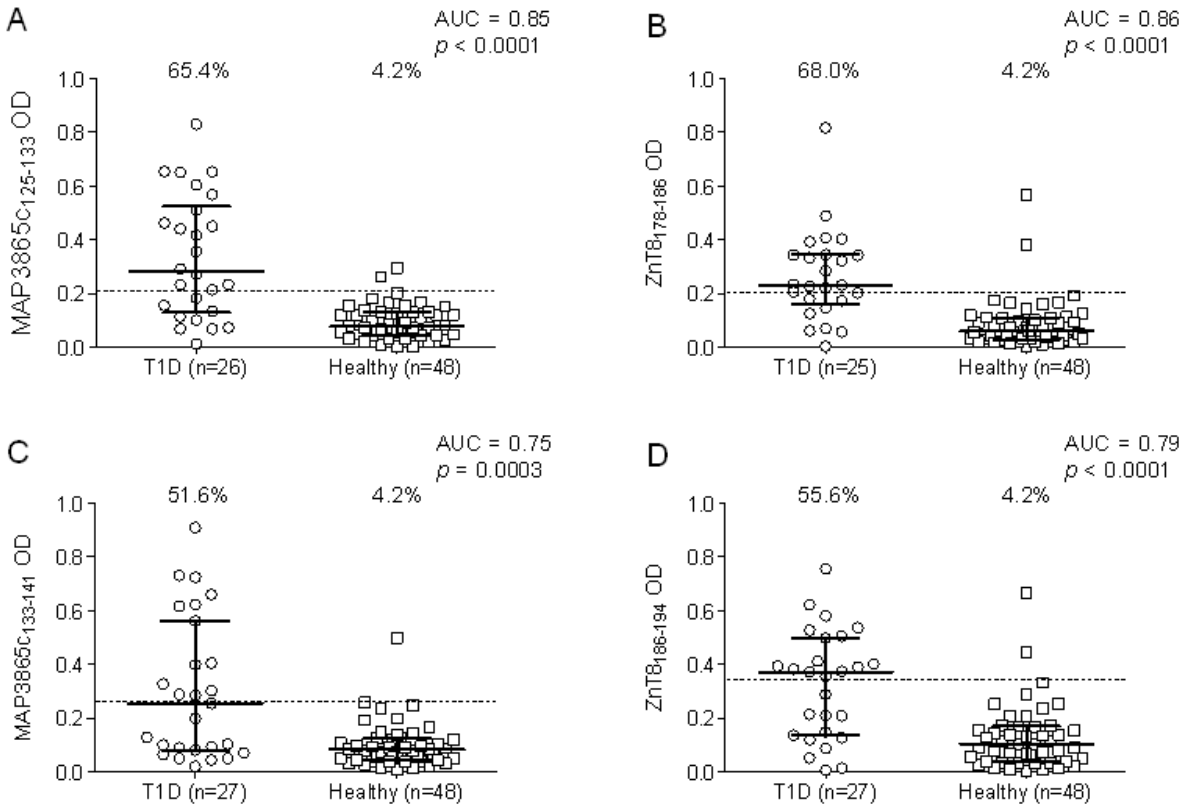

Fig. 15. Prevalence of Abs against MAP3865c$_{125\text{-}133}$ (A) and its homologous ZnT8$_{178\text{-}186}$ (B); and against MAP3865c$_{133\text{-}141}$ (C) and its homologous ZnT8$_{186\text{-}194}$ (D) in T1D and healthy subjects. Data representation is the same as in Fig. 9.

As observed for the whole MAP3865c protein, this reactivity was specific of T1D patients, as it was not observed among T2D subjects (Fig. 16).

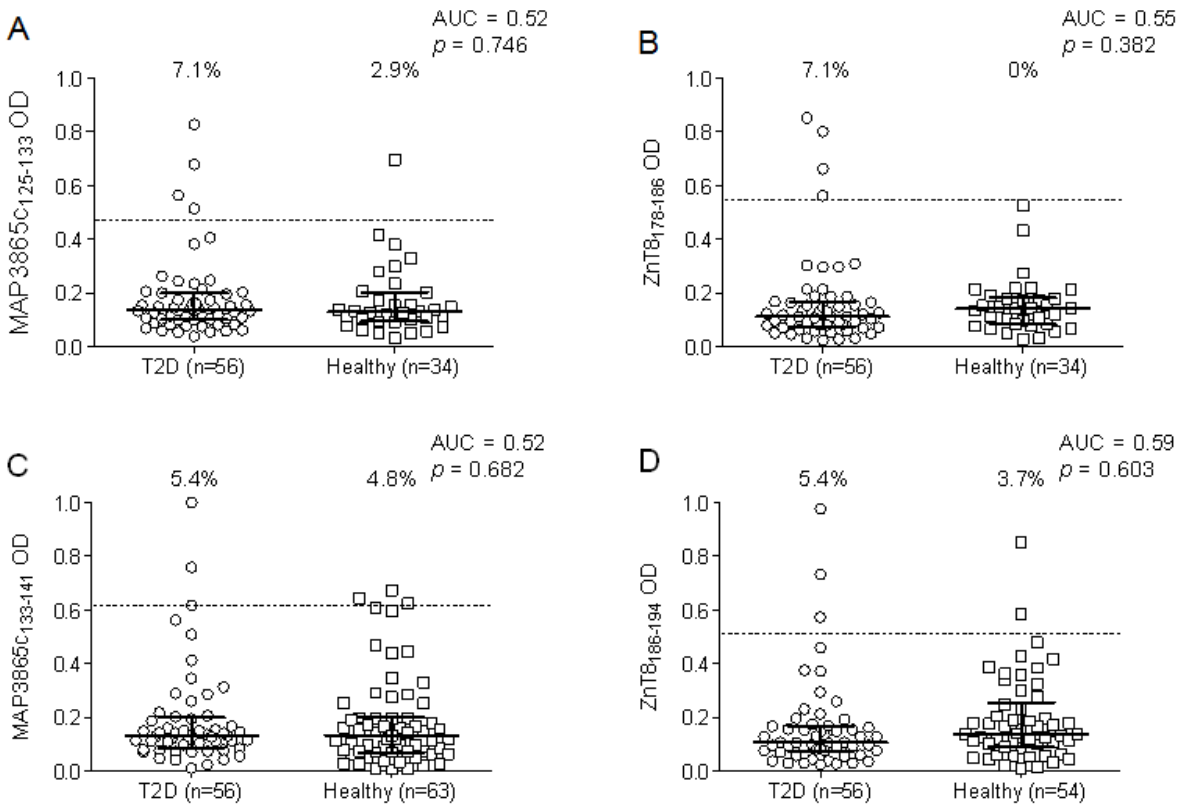

Fig. 16. Prevalence of Abs against MAP3865c$_{125\text{-}133}$ (A) and its homologous ZnT8$_{178\text{-}186}$ (B); and against MAP3865c$_{133\text{-}141}$ (C) and its homologous ZnT8$_{186\text{-}194}$ (D) in T2D and healthy subjects. Data representation is the same as in Fig. 9.

Comparison of Ab+ and Ab-negative T1D patients (Table 4) showed that anti-MAP3865c Ab+ patients had a significantly shorter disease duration than Ab-negative pairs (10.9±7.7 vs. 17.9±10.0; p=0.025). Similar trends were observed when comparing T1D patients harboring or not Abs against MAP3865c$_{125\text{-}133}$ (12.6±8.8 vs. 17.2±10.0; p=0.068) and its homologous ZnT8$_{178\text{-}186}$ (12.6±8.8 *vs.* 18.0±10.0; p=0.068), but not for Abs against MAP3865c$_{133\text{-}141}$ (13.2±8.6 *vs.* 17.5±10.4; p=0.170) or its homologous ZnT8$_{186\text{-}194}$ (13.8±12.3 *vs.* 17.5±9.5; p=0.296). A trend towards an older age at T1D diagnosis was also observed in patients positive for Abs against MAP3865c (22.9±9.6 *vs.* 16.3±10.0; p=0.072).

	T1D duration (yrs)	Age at T1D diagnosis (yrs)
MAP3865c Ab+	10.9 ± 7.7	22.9 ± 9.6
MAP3865c Ab-	17.9 ± 10.0	16.3 ± 10.0
p	0.025	0.072
MAP3865c$_{125-133}$ Ab+	12.6 ± 8.8	19.8 ± 11.0
MAP3865c$_{125-133}$ Ab-	17.2 ± 10.0	17.1 ± 9.8
p	0.068	0.340
ZnT8$_{178-186}$ Ab+	12.6 ± 8.8	19.8 ± 11.0
ZnT8$_{178-186}$ Ab-	18.0 ± 10.0	16.7 ± 9.8
p	0.068	0.340
MAP3865c$_{133-141}$ Ab+	13.2 ± 8.6	20.8 ± 10.2
MAP3865c$_{133-141}$ Ab-	17.5 ± 10.4	16.6 ± 10.5
p	0.170	0.229
ZnT8$_{186-194}$ Ab+	13.8 ± 12.3	20.1 ± 14.3
ZnT8$_{186-194}$ Ab-	17.5 ± 9.5	16.6 ± 9.3
p	0.296	0.347

Table 4. T1D duration and age at T1D diagnosis in Ab+ and Ab-negative T1D patients. T1D patients whose Ab reactivities are shown in Figures 9 and 15 were compared using the Mann-Whitney U test. Mean ± SD are shown.

4.8 Anti-MAP3865c and anti-ZnT8 Abs recognizing homologous sequences are cross-reactive

The similar frequencies of Abs recognizing MAP3865c and ZnT8 homologous regions among T1D patients (65.4-68.0% and 51.6-55.6%, respectively; Fig. 15) suggests that Abs targeting these regions could be cross-reactive. Indeed, there was a high degree of correlation between the titers of Abs recognizing MAP3865c and ZnT8 homologous sequences in both T1D patients and healthy controls (Fig. 17A-B; r^2=0.74 for MAP3865c$_{125-133}$ *vs.* ZnT8$_{178-186}$ and r^2=0.58 for MAP3865c$_{133-141}$ *vs.* ZnT8$_{186-194}$; p<0.0001). This correlation was maintained

when the analysis was restricted to either T1D patients or healthy controls (data not shown). This demonstrates that anti-MAP3865c and anti-ZnT8 Abs recognizing homologous sequences segregate within the same sera. The same was true for Ab reactivities against the two neighboring regions MAP3865c$_{125\text{-}133}$ and MAP3865c$_{133\text{-}141}$ and for ZnT8$_{178\text{-}186}$ and ZnT8$_{186\text{-}194}$ (Fig. 17C-D; r^2=0.67 and 0.74, respectively; p<0.0001), suggesting that recognition of these peptides stems from an immune responses against the whole MAP3865c/ZnT8 transmembrane region to which they belong.

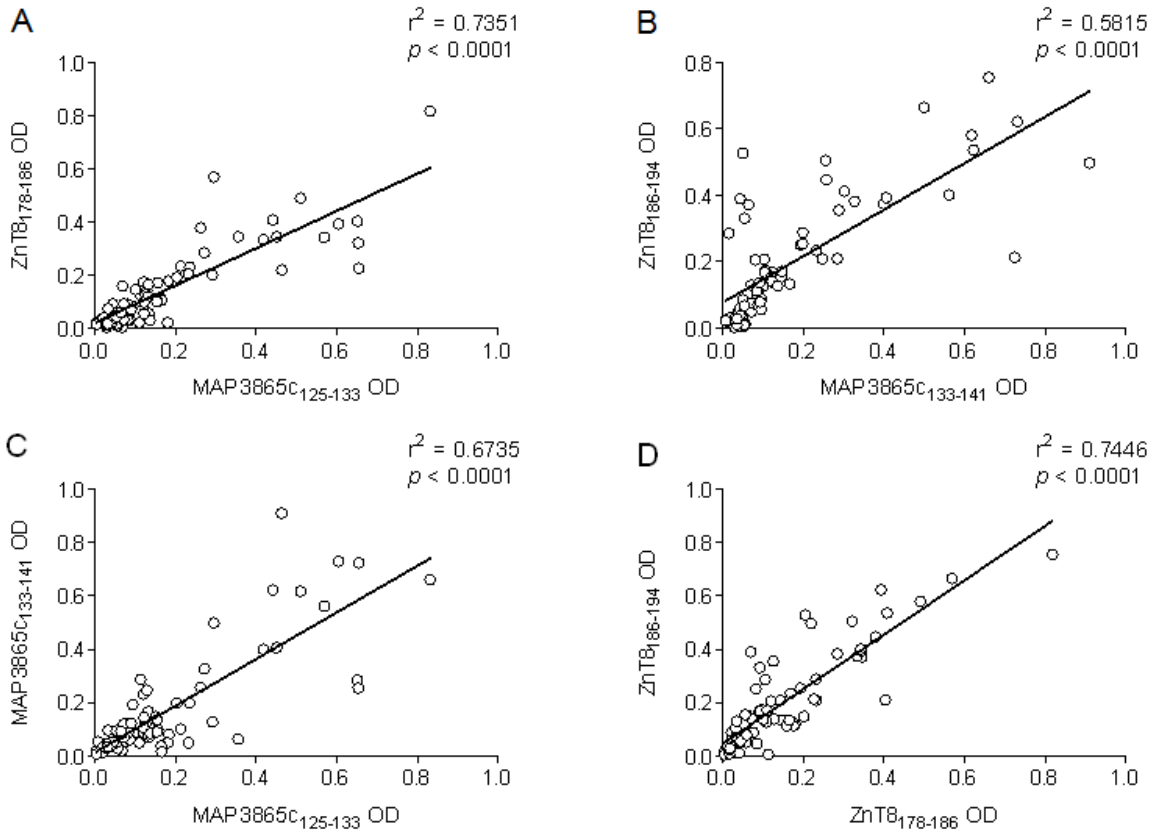

Fig. 17. Correlation between titers of MAP3865c- and ZnT8-reactive Abs recognizing different epitopes. Correlations are shown between titers of Abs recognizing (A) MAP3865c$_{125\text{-}133}$ and its homologous ZnT8$_{178\text{-}186}$ epitope; (B) MAP3865c133-141 and its homologous ZnT8$_{186\text{-}194}$ epitope; (C) MAP3865c$_{125\text{-}133}$ and its consecutive MAP3865c$_{133\text{-}141}$ epitope; (D) ZnT8$_{178\text{-}186}$ and its consecutive ZnT8$_{186\text{-}194}$ epitope. Each circle represents the titers of one T1D or healthy donor.

To verify whether co-segregation of these reactivities was due to Ab specificities cross-reacting between each other, competition experiments were performed. Anti-MAP3865c$_{125-133}$-positive and -negative sera were pre-adsorbed overnight with different peptides, and then subjected to ELISA assays on MAP3865c$_{125-133}$-coated plates (Fig. 18A). While a control peptide did not cause any decrease in signal, both MAP3865c$_{125-133}$ and its homologous ZnT8$_{178-186}$ peptide strongly inhibited the MAP3865c$_{125-133}$ reactivity to a similar extent (57-89%). The same observation was repeated with the MAP3865c$_{133-141}$ reactivity, which was efficiently inhibited (55-66%) upon serum pre-adsorption with either MAP3865c$_{133-141}$ or its homologous ZnT8$_{186-194}$ (Fig. 18B). Taken together, these results demonstrate that anti-MAP and anti-ZnT8 Abs targeting homologous membrane-spanning sequences are cross-reactive.

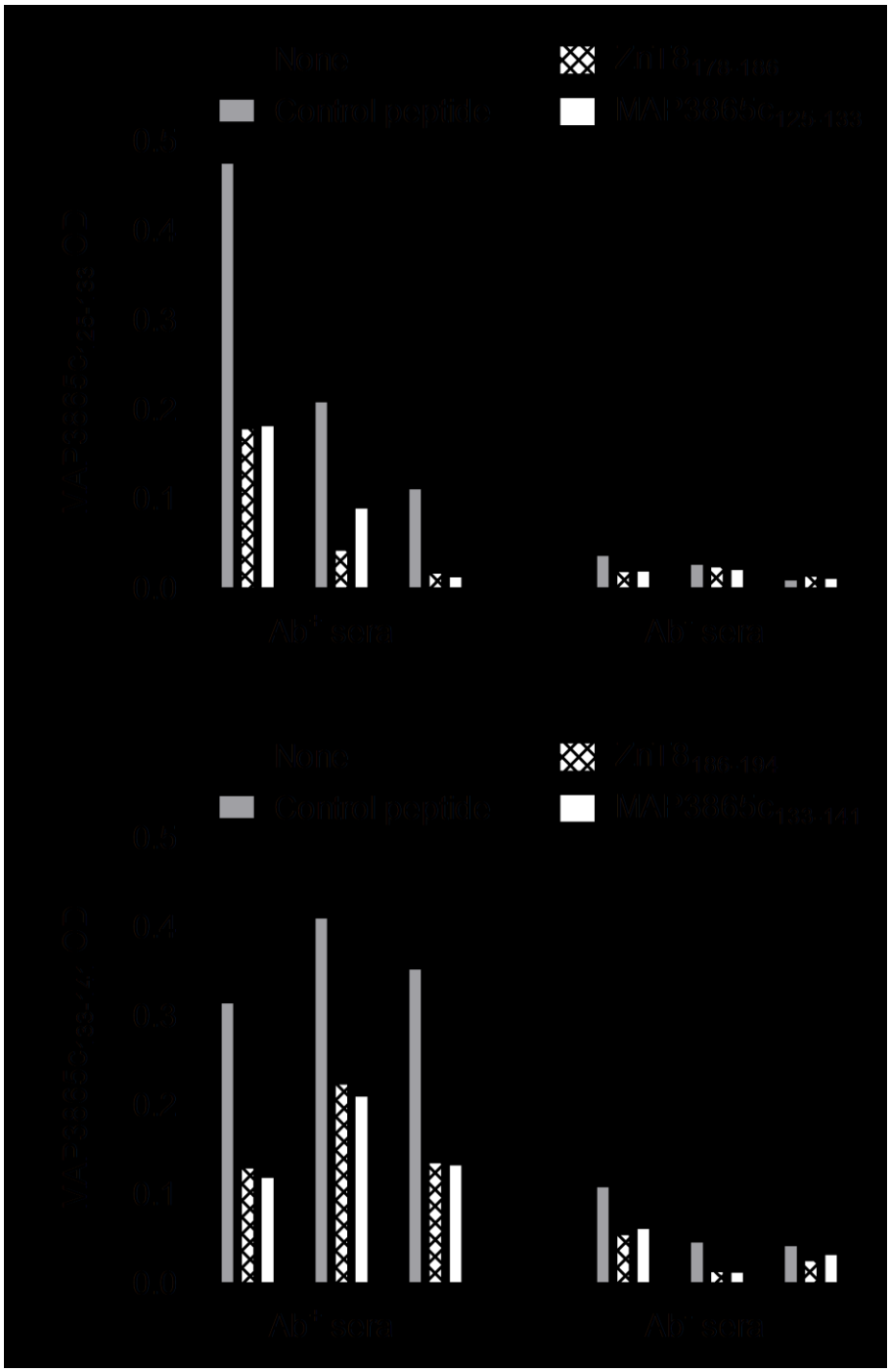

Fig. 18. Ab reactivities against MAP3865c epitopes are inhibited by the homologous ZnT8 epitopes. (A) Ab+ and Ab-negative sera from T1D patients were pre-incubated overnight with saturating concentrations of MAP3865c$_{125\text{-}133}$ (white bars), ZnT8$_{178\text{-}186}$ (hatched bars), control

(grey bars) or no peptide (black bars) and their reactivity on MAP3865c$_{125-133}$-coated ELISA plates subsequently tested. (B) The same sera were preincubated with MAP3865c$_{133-141}$ (white bars), ZnT8$_{186-194}$ (hatched bars), control (grey bars) or no peptide (black bars) and their reactivity on MAP3865c$_{133-141}$-coated ELISA plates subsequently tested. Bars depict means ± SEM of triplicate wells and results are representative of two separate experiments.

5 Discussion

Firstly, it was successfully demonstrated the presence of MAP's DNA in T1D Sardinian cases, this goal was achieved through PCR based detection of IS900 insertion element, a specific signature locus of MAP (Sechi *et al*., 2008). After identifying MAP in the blood of T1D and aiming to understand the host immune responses to MAP, it was designed an immunoassays (indirect ELISA) for the detection of anti-MAP antibodies in diabetic patients. ELISA tests, employing sensitive antigenic targets such as HbHa (heparin binding hemagglutinin) and Gsd (glycosyl transferase) proteins, gave encouraging results (Sechi, Rosu *et al.,* 2008).

However, anti-MAP humoral responses corresponding to HbHa and Gsd could not be indicative of an active infection and also since these proteins are encoded by wider range of mycobacteria, this raised an issue of cross-reactivity with tubercle bacilli which could be an issue to deal with the BCG vaccinated individuals. This prompted Sechi *et al.* to set up an immunoassays including a MAP specific protein, MptD into the battery of antigens. The detection of anti-MAP antibodies revealed extremely significant humoral immune responses in T1D patients when compared to T2D and healthy controls (Rosu *et al.,* 2009, Cossu A. *et al.,* 2011). A final evidence supporting a link between MAP and T1D was presented in terms of culture of MAP bacilli from the blood of two of the T1D patients from Sardinia (Rosu *et al.,* 2009). Summing up, different Map proteins were shown to be highly recognized in T1D patients and even if some of them were specific of Map (MAP3738c and MptD) none of them were homologous to human proteins. It was compelling to search a possible explanation of the association between MAP and T1D. So it was blasted the whole Map genome against the human genome and it was identified a protein (MAP 3865c) not only homologus to human proteins but also specific to β-cells (Znt8).

Building up on the above reports documenting a high prevalence of MAP infection and seroreactivity in Sardinian T1D patients (Sechi *et al.,* 2008; Paccagnini *et al.,* 2009; Rosu *et al.,* 2008; Cossu A. *et al.,* 2011) it was here demonstrated that the MAP3865c protein is a target of Ab responses that cross-react with homologous ZnT8 sequences. MAP3865c is a

298 aminoacid 6-membrane-spanning channel which endows MAP with the ability to transport cations through the membrane, an important feature associated with intracellular survival of mycobacteria (Riccardi *et al.,* 2008). ZnT8 is a 369 aminoacid protein which belongs to the cation diffusion facilitator family of highly homologous ZnT (Slc30) proteins. It displays a remarkably similar structure and function, allowing Zn^{2+} to accumulate in the insulin granules of pancreatic β-cells. Zn^{2+} cations are essential to form hexavalent insulin storage crystals and, eventually, for effective insulin secretion (Wijesekara *et al.,* 2010). Most of the 71 aminoacid difference in length between MAP3865c and ZnT8 is made up by the first extra-luminal domain, which is much shorter for MAP3865c (Fig. 13B). To look for potential cross-reactive Ab epitopes, the analysis was focused on a trasmembrane region of high homology. Ab reactivities against peptide sequences of this region were even more prevalent in T1D patients than those against the whole MAP3865c protein, perhaps reflecting masking of these hydrophobic epitopes in the solubilized MAP3865c protein. Importantly, Abs against this membrane-spanning epitopes would not be detected by conventional anti-ZnT8 aAb assays, which employ a fusion protein combining the 4 extraluminal domains of ZnT8 (Wenzlau, Juhl *et al.,* 2007). Other regions of high homology are mostly located in these extra-luminal domains, raising the possibility that other cross-reactive epitopes may be recognized by other Abs, including conventional anti-ZnT8 aAbs. Of further note, the transmembrane region identified here does not comprise the polymorphic ZnT8 R/W variant at position 325, which is located in the last extra-luminal domain, thus making it unlikely that the ZnT8 genetic background may shape these Ab reactivities, as described for conventional anti-ZnT8 aAbs (Wenzlau, Liu *et al.,* 2008).

The intestinal localization of MAP infection may also favor cross-reactivity with Abs and T cells recognizing ZnT8.

The primary route of MAP infection is fecal- oral and once ingested, the bacterium lodges in to the mucosa associated lymphoid tissue (MALT) of the small intestine. It is then endocytosed by the M cells of Peyer's patches, which are further phagocytosed by intra epithelial macrophages.

Indeed, the first encounter with beta-cell antigens takes place in pancreatic lymph nodes (Gagneraul *et al.,* 2002), which also drain intestinal tissues (Turley *et al.,* 2005). Epitope mimicry and spreading may be further favored by high precursor frequencies of ZnT8-reactive naïve T cells. As ZnT8 has not been found expressed by medullary thymic epithelial cells (Derbinski *et al.,* 2005), negative selection of ZnT8-reactive T cells may be ineffective. Thus, tolerance to ZnT8 may heavily rely on peripheral mechanisms such as immune ignorance, which may be readily overcome by MAP infection.

The intestinal localization of MAP infection may also give reason for the lack of correlation between MAP IS900 DNA and Ab detection. Not all MAP-infected individuals may mount systemic Ab responses detectable in blood, or they may develop Abs against other MAP antigens.

It is still premature to conclude whether MAP3865c-ZnT8 cross-reactivity implies an epitope mimicry phenomenon initiating or precipitating T1D. To this end, three key points remain to be addressed. First, MAP3865c-ZnT8 cross-reactivity was documented at the Ab level, but we did not explore potentially cross-reactive T-cell responses. CD4+ T-cell priming is an early event in the autoimmune cascade, providing help to B lymphocytes for differentiating into Ab-secreting plasma cells. Importantly, none of the ZnT8-derived $CD4^+$ T-cell epitopes recently described (Dang *et al*., 2011) map to the Ab-targeted region here identified. Moreover, $CD8^+$ T cells are additional key players in beta-cell autoimmunity (Mallone *et al*., 2007). Characterization of ZnT8- and MAP3865c-specific $CD8^+$ T-cell responses using technologies described elsewhere (Martinuzzi *et al.,* 2008) is currently in progress. Second, is MAP infection and sero-reactivity already present before or at T1D onset, i.e. in at risk and new-onset T1D subjects? Once T1D established, we cannot resolve whether MAP infection preceded or followed T1D onset, pointing either to a causal relationship with disease or to a consequence thereof.

The observed correlation between anti-MAP Abs and shorter disease duration makes the first hypothesis more attractive. It may be argued that anti-ZnT8 aAbs typically appear late in at-risk children (Wenzlau, Juhl *et al.,* 2007), following the first aAb markers of beta-cell autoimmunity. If anti-ZnT8 aAbs are generated through MAP epitope mimicry, this would

then suggest a late appearance of MAP infection. However, such late appearance would rule out an initiating effect, but not a precipitating contribution to T1D development once autoimmunity has been initiated, as proposed for coxsackie virus infections (Serreze *et al.,* 2000, Filippi *et al.,* 2008). Moreover, the T1D patients studied here were adults, for whom the hierarchy of aAb appearance may not be the same as for children. Indeed, adult T1D patients are rarely insulin aAb+ and more frequently harbor anti-GAD and/or anti-IA-2 aAbs, which do not clearly preceed anti-ZnT8 aAbs (Wenzlau, Juhl *et al.,* 2007).

Of further note, the relationship between the classical anti-ZnT8 aAbs and MAP cross-reactivity remains to be established, as the cross-reactive epitopes here identified are located outside the regions recognized by these aAbs. It is thus possible that early MAP infection may initially ignite immune responses against this transmembrane region, to only later spread to the extra-luminal epitopes covered by anti-ZnT8 aAb assays. Third, it is well established that T cells are the key pathogenic effectors of T1D, while aAbs only play an accessory role which is matter of debate (Mallone *et al.,* 2011).

It is here postulated that antibodies production against ZNT8 do contribute to the T1D pathogenesis but in a small part (e.g. antibodies may act as ligands activating macrophages contributing to the tissue inflammation). The main part should be due to the development of a Th1-Th17 response stimulated by the MAP3865c and successively by the ZNT8 protein that may initiate and continue β-cells destruction.

Therefore, to definitely prove a causal relationship beyond the correlation between MAP infection and T1D, mouse studies should be performed to document that MAP infection, MAP3865c immunization and/or MAP-reactive T cells induce disease. These experiments are difficult to perform for a number of reasons. The presence of ZnT8-specific aAbs or T cells have not yet been reported in NOD mice. Moreover, the Ab epitopes here identified are poorly conserved in the mouse. Of further note, we cannot discount the possibility of a direct cytotoxic effect of anti-MAP3865c/ZnT8 cross-reactive Abs through binding to β-cells.

The transmembrane location of these epitopes may allow Abs to exert an agonistic or antagonistic effect on Zn^{2+} transport through the ZnT8 channel, possibly impinging on the β-cell secretory capacity.

The majority – but not all (Bruno *et al.,* 1996) – of studies on Sardinian migrants suggests a predominant weight of genetic factors on T1D pathogenesis, as disease incidence in the hosting region remains similar to that of Sardinia (Muntoni *et al.,* 1997). This observation is at variance with most T1D migrant studies (Bodansky *et al.,* 1992) and with comparisons of T1D incidence between neighboring regions of uniform genetic background and diverse environmental exposure (Kondrashova *et al.,* 2007), which instead point to a prominent role of environmental factors. The possible involvement of MAP in the pathogenesis of Sardinian T1D may reconcile this conundrum. As MAP has been shown to pass into human breast milk (Naser *et al.,* 2000), children born outside Sardinia from Sardinian mothers could still be more exposed to MAP infection. Moreover, Sardinian migrants frequently maintain regular interactions with their homeland relatives, including exchange of regional dairy and meat products which may contribute in maintaining MAP exposure. Another open question is whether our current observations are specific to the Sardinian population or can be replicated elsewhere. Although MAP exposure is particularly high in Sardinia, contamination of food products with MAP has been documented worldwide. While we are not aware of studies performed in other areas at very high T1D incidence such as Scandinavia, studies conducted in UK document a MAP prevalence in herds and dairy products similar to what found in lower incidence countries (Eltholth *et al.,* 2009). Thus, the environmental prevalence of MAP does not systematically parallel that of T1D, suggesting that other factors may be at play. As suggested for enteroviral infections (Serreze *et al.,* 2000, Filippi *et al.,* 2008), timing of MAP infection may be one such factor, making epitope mimicry effective only once an inflammatory milieu is established, once β-cell autoimmunity is initiated and/or once a critical mass of ZnT8-reactive T cells have already accumulated. The chronic nature of MAP infection may also perpetuate availability of cross-reactive epitopes. Ultimately, the interaction between MAP environmental exposure and a susceptible genetic background such as the Sardinian one may be critical, as children whose parents have migrated to Sardinia do not acquire a higher T1D risk (Frongia *et al.,* 2001).

Indeed, the gene pool of Sardinians is highly distinct from that of all other Mediterranean regions, probably reflecting a genetic drift entrenched over centuries by geographical

isolation, epidemic misfortunes and endogamy (Calo *et al.,* 2008). Sardinia, with alleles and haplotypes that are rare or absent elsewhere poses as a high endemic zone of autoimmune disorders such as T1D, lupus erythematosus and multiple sclerosis. An interplay of genetic and environmental factors in Sardinia possibly could make it a hotspot to study autoimmune disorders.

Genetic susceptibility may be conferred by polymorphic loci involved in clearance of intracellular bacteria, such as the *SLC11A1* locus (previously known as *NRAMP1*). *SLC11A1* polymorphisms have been associated with T1D in Sardinian (Paccagnini *et al.,* 2009) and Japanese (Takahashi *et al.,* 2004) patients and in NOD mice (Kissler *et al.,* 2006), as well as with susceptibility to mycobacterial infections (Bellamy *et al.,* 1998).

In humans, SLC11A1 – solute carrier 11A1 gene is mapped on 2q35, is composed of 15 exons and it spans at least 16 kb of the DNA. It encodes an integral membrane protein of 550 amino acids localized in the late endosomal/lysosomal compartments of phagocytes, where it functions as a transporter of divalent cations such as Zn^{2+} (Jabado *et al.,* 2000).

Transport of divalent cations may deprive mycobacteria of these important elements (Riccardi *et al.*, 2008) and contributes to enhanced phagosomal acidification (Dai *et al.,* 2009), thus impairing survival of intracellular pathogens (Jabado *et al.,* 2000) and modifying processing and presentation of certain epitopes, including β-cell derived ones (Dai *et al.,* 2009). Another intriguing observation is that the long-standing diatribe as to whether early childhood exposure to cow's milk predisposes to islet Ab seroconversion and T1D has remained unsettled (Norris *et al.,* 1996, Knip *et al.,* 2000). MAP contamination of milk supplies and the interaction between MAP environmental exposure and a susceptible genetic ground may offer an alternative key of interpretation for further investigations. Finally, the individual genetic background over which MAP exposure builds up may dictate susceptibility to other autoimmune and inflammatory diseases such as MS and Crohn's disease, which have also been associated with MAP infection in Sardinia (Scanu *et al.,* 2005, Cossu D. *et al.,* 2011) and, in the case of Crohn's disease, elsewhere (Naser *et al.,* 2004).

Further investigations outside Sardinia and comprehensive dissection of the groove between genetic and environmental factors will be critical in deciphering the significance of these findings in T1D pathogenesis.

6 Conclusion

Just because MAP is detected more often in the blood of T1D patients by culture, PCR or ELISA than in control subjects does not necessarily mean that MAP causes T1D. This is certainly evidence of an association (the occurrence at the same time and in the same patient of MAP T1D) but not necessarily of causation (the organism has directly initiated the disease in the patient). There are several possible explanations for the presence of MAP in the blood of T1D patients: it could be an innocent bystander that has merely colonized the host; it could be a secondary infection but not causing the disease; or it could be the primary infectious agent and the cause of T1D. Definitive evidence proving a causal relationship between MAP and T1D is not available at present, perhaps it may never be. However, there is evidence suggesting some kind of association between MAP and at least some cases of T1D, so much that the robustness of the link is undeniable. The main push for now is to obtain data supporting a direct involvement of MAP in triggering the disease. Here below it is postulated a possible mechanism underlying MAP induced β-cell destruction.

Briefly if MAP was to have a role in the pathogenesis of T1D then it is possible that the infection is food or waterborne. The most likely candidates as vehicles of transmission of MAP from cattle to humans are milk (and potentially other dairy products), beef and water. Once MAP is ingested, it resides and colonizes the MALT of the small intestine. It is then endocytosed by the M cells of Peyer's patches, which are further phagocytosed by intra epithelial macrophages. MAP persistence in the gut might cause the spreading of inflammatory mediators responsible in turns of initiating inflammation in the pancreas. Activated macrophages, acting as Antigen Presenting Cell (APC), can engulf MAP and enable it to reach pancreatic lymphonodes. Once in there macrophages process and expose MAP's epitopes on their surface receptors. These events may activacte cross reactive $CD8^+$ T cells and $CD4^+$ T cells which induce β-cell destruction mainly through massive production of pro-inflammatory cytokines and chemokines. These soluble mediators as matter of fact orchestrate the immune response, attracting $CD4^+$ effector T cells, accounting for the insulitis, and driving the polarization of the immune response towards a Th1-Th17 phenotype.

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8 Abbreviations

aAbs, autoantibodies;

Abs, antibodies;

AIRE, Autoimmune regulator transcription factor;

APC, antigen-presenting cells;

BB-DP , biobreeding diabetes-prone;

CTLA-4, Cytotoxic T lymphocyte associated antigen 4;

CD, Crohn's disease;

DC, dendritic cells;

ELISA, enzyme-linked immunosorbent assay;

FAP, fibronectin attachment protein;

GAD65, glutamic acid decarboxylase;

GABA ,γ-amino butyric acid;

GD, Gestational diabetes;

HLA, human leukocyte antigen;

IAA, Insulin autoantibodies;

IA-2, tyrosine phosphatase-like insulinoma antigen-2;

IPTG, isopropyl-β-D-thiogalactopyranoside;

JD, Johne's disease;

LADA, latent autoimmune diabetes in adults;

MS, multiple sclerosis;

MALT, mucosa associated lymphoid tissue;

MAP, *Mycobacterium avium* subspecies*paratuberculosis*;

MBP, maltose-binding protein;

MHC I-II, major histocompability complex I-II;

MODY, maturity onset diabetes in the young;

MS, multiple sclerosis;

NK, natural killer;

NOD, non-obese diabetic;

NRAMP1, Natural Resistance-Associated Macrophage Protein;

OD, optical density;

PBS-T, PBS-Tween 20;

ROC, receiver operator characteristic;

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis;

T1D, type 1 diabetes;

T2D, type 2 diabetes;

TCR, T-cell receptor;

Treg, regulatory T cells;

ZnT8, zinc transporter 8;

VNTR, variable number of tandem repeat;

WHO, World Health Organization;